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Trace elements quantification in Portuguese red wines

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Abstract

The aim of this thesis is to characterize Portuguese red wines in terms of trace elements composition. The wines were chosen so that all the country was represented and studied.

For trace elements quantification (As, Hg, Cd, Ni and Pb) were tested various sample treatments including for all trace elements: acid digestion or presence and absence of spike. The need for H_2O_2 addition in order to oxidize organic compounds was analyzed for Hg, Cd, Ni and Pb. Quantification of all trace elements was performed with Atomic Absorption Spectrometry techniques. After the method validation were analyzed 25 Portuguese red wines and duplicates. The concentrations obtained were used to perform a statistical analysis to determine what were the regions with highest incidence of each trace element. Using Target Hazard Quotient (THQ) equation was possible to identify the regions where the concentrations found are a reason for public health concern, being values above 1 a motive for concern.

After the analysis was determined that there is no need for wine samples digestion and that the presence of H_2O_2 is crucial. Hg and As were quantified with Hydride Generation Atomic Absorption Spectrometry; Ni and Pb with Flame Atomic Absorption Spectrometry; Cd with Electrothermal Atomic Absorption Spectrometry.

The statistical results allowed to conclude that the system variation was mainly explained by the variation of Ni, As and Hg concentrations. Ni was largely found in Estremadura and Terras do Sado wines, while As and Hg were found mostly in Minho and Douro wines respectively.

All of THQs determined were under 1, which is the limit value above which there is reason for health concern. Maximum THQ values were of 0.044 in Algarve wines were due to Ni.

Keywords: Portuguese red wines; trace elements; Atomic Absorption Spectrometry; Target Hazard Quotients.

Resumo

O objectivo desta tese é caracterizar os vinhos tintos Portugueses em termos de composição em metais pesados e matalóides. Os vinhos foram escolhidos de modo a que todo o País esteja representado e seja estudado.

Para quantificar os metais pesados e metalóides (As, Hg, Cd, Ni e Pb) testaram-se vários tratamentos de modo a determinar quais deveriam ser efectuados às amostras antes da quantificação. Os estudos de pré-tratamento incluiram para todos os metais pesados e metalóides: digestão ácida, presença e ausência de dopagem. A necessidade da presença de H_2O_2, para oxidar compostos orgânicos, foi analisada para Hg, Cd, Ni e Pb. A quantificação dos elementos realizou-se com técnicas de Espectrometria de Absorção Atómica. Após a validação do método analisaram-se 25 vinhos tintos Portugueses e duplicados. Recorrendo às concentrações obtidas realizou-se uma análise estatística para determinar as regiões com maior incidência de cada elemento. Através da equação que determina os "Target Hazard Quotiente" (THQ) foi possível identificar as regiões onde as concentrações encontradas são motivo de preocupação, sendo que valores acima de 1 são considerados motivo de preocupação.

Durante a validação do método determinou-se que não existe necessidade de digerir as amostras e que a presença de H_2O_2 é crucial. Hg e As foram quantificados com Espectrometria de Absorção Atómica por Geração de Hidretos; Ni e Pb com Espectrometria de Absorção Atómica por Chama; Cd com Espectrometria de Absorção Atómica por Forno de Grafite.

Dos resultados estatísticos concluiu-se que a variação do sistema pode ser explicada pela variação de concentrações de Ni, As e Hg. O Ni foi encontrado maioritariamente em vinhos da Estremadura e Terras do Sado, enquanto As e Hg foram encontrados principalmente em vinhos do Minho e Douro respectivamente.

Todos os THQs revelaram valores inferiores a 1, tendo o valor máximo 0,044 para vinhos do Algarve devido ao Ni.

Palavras-chave: vinho tinto Português; metais e metalóides; Espectrometria de Absorção Atómica; "Target Hazard Quotient".

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List of abbreviations

AAS - Atomic Absorption Spectrometry

CE – European Community

DIL - Daily Intake Levels

DL – Detection Limit

DO – Designation of Origin

DOC – Controled Designation of Origin

ED_{tot} - Exposure duration (year)

EF_r - Exposure frequency (days $year^{-1}$)

EPA – Environmental Protection Agency

ETAAS - Electrothermal Atomic Absorption Spectrometry

EU – European Union

FAO/WHO - Food and Agriculture Organization/World Health Organization

FAAS - Flame Atomic Absorption Spectrometry

HGAAS – Hydride Generation Atomic Absorption Spectrometry.

IARC – International Agency for Research on Cancer

ICP - Inductively Coupled Plasma

ICP-MS - Inductively Coupled Plasma Mass Spectrometry

IG – Geographical Indication

IGP – Protected Geographical Indication

INE – National Institute of Statistics

IVV - Portuguese Institute of Vine and Wine

OECD - Organisation for Economic Co-operation and Development (OCDE)

OIV - Organisation Internationale de la Vigne et du Vin

PCA – Principal Component Analysis

PTWI – Provisional Tolerable Weekly Intake

RDI - Reference Daily Intake

Rec – Recovery

RSD - Relative Sandard Deviation

THQ - Target Hazard Quotients

UL – Upper Intake Level

WAD – With acid digestion

WD - Without acid digestion

Mathematical notations

ε - Absorbtivity

AT_c - Average time for carcinogenic trace elements (days)

AT_n - Average time for non-carcinogenic trace elements (days)

BW_a - Body weight (kg)

MCS_{inorg} - Concentration of inorganic species (µg g^{-1})

R^2 - Correlation coefficients

g - Gram

hL – Hectolitre

kg – Kilogram

L – Liter

SFI - Mass of the selected dietary ingested (g day^{-1})

µg – Microgram

mL – Mililitre

mg – Milligram

RfD - Oral reference dose (mg kg^{-1} day^{-1})

b – Path length (cm)

1. Introduction

1.1 Wine National Production

Wine is defined in European Union (EU) as "the product obtained exclusively from the total or partial alcoholic fermentation of fresh grapes, whether crashed or not crushed, or of grape must", according to the Annex 1 of the Regulation 1493/1999.

Wine has been consumed worldwide for thousands of years in large scale. The Portuguese current consumption is above 4568×10^5 L per year according to the National Institute of Statistics (INE, 2008), which is of great relevance to the Portuguese economy. Wines of high quality are produced in this country, some being even recognized all over the world (Curvelo-Garcia, 1988).

Portugal has almost 246×10^3 ha of cultivated area (2008 as the reference year), which represents the fourth place on the European production area ranking (Table 1.1), only being overpassed by Spain, France and Italy. The data shown in Table 1.1 is from three years ago because the values for 2009 are still provisional and for 2010 are forecasted. So, in order to maintain accuracy, the year of 2008 was chosen because data is entirely confirmed by the Organisation Internationale de la Vigne et du Vin (OIV) (2011).

Table 1.1: Wine areas in Europe in 2008 (OIV, 2011).

Country	**Wine areas (10^3 ha)**
Spain	1165
France	852
Italy	825
Portugal	246
Greece	116
Germany	102
Austria	48
Total	**3354**

The 246×10^3 ha of planted vineyards have originated 5412 thousand hL of wine in Portugal, from which 1189 thousand hL was red wine (INE, 2008).

1.2 Wine characteristics

Wine composition depends upon various parameters, namely: soil, climate, castes and winemaking process (Dessuy *et al.*, 2007; Voica *et al.*, 2009).

- Soil components, such as water, nutrients or minerals, influence not only the development of vineyard, but also the final grape composition. Soils with negative charges in the surface particles attract cations. pH also influences the absorption of minerals, because it modifies the solubility of those elements. Soils constituted of clay and humus, have on the surface negative charges that attract cations and they remain absorbed into the soil. In contrast, sandy soils do not have characteristics needed to fix the minerals, so they are easily leached by rainfall or irrigation.

- Climate influences mostly the process of maturation, for which is needed a certain time of exposition to sun. The rain is needed during the spring, but not in the last part of the cultivation season for a complete maturation.

- Castes are responsible mainly for flavor characteristics of wine.

- Winemaking process can change the final product, especially in red wine production, due to the presence of grape skin. Final product is influenced by the extension of each step in the process (Figure 1.1) (Garrido *et al.*, 1997).

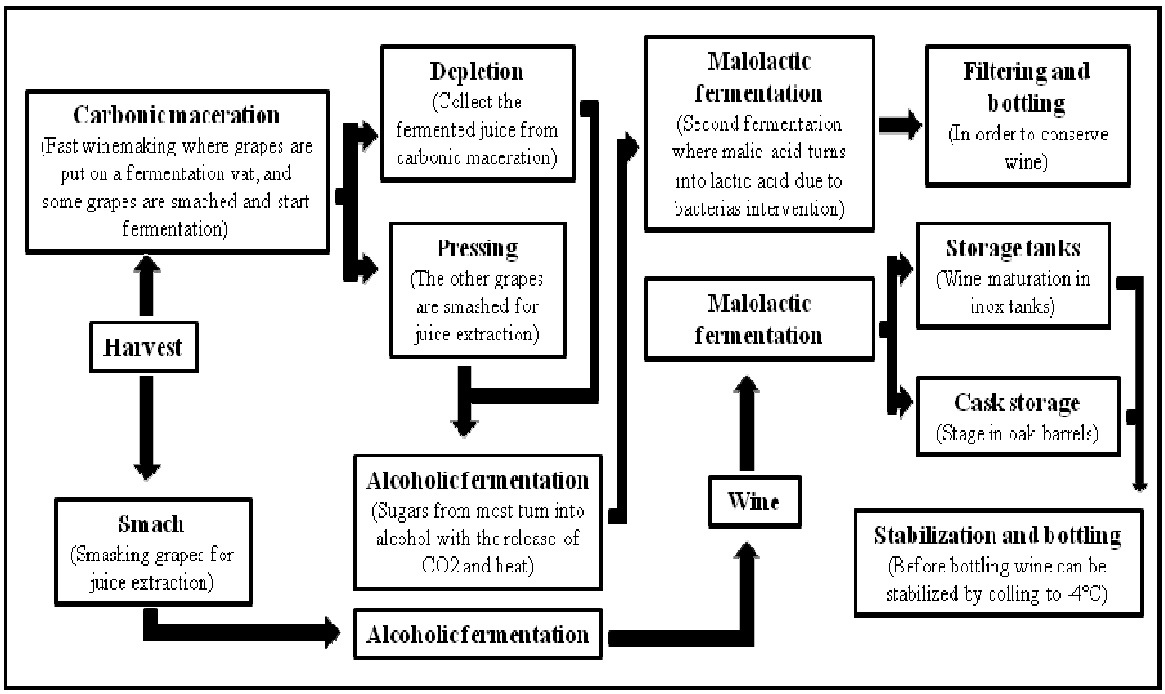

Figure 1.1: Portuguese red wine production (Coutinho, 2010).

Wine is a very complex matrix that is composed by alcohol, water, sugar, organic and inorganic compounds which determine different characteristics, for instance, color, aroma and flavor (Riviero-Pérez *et al.*, 2008). Organic compounds that make part of wine can be divided in two groups: volatile and non-volatile compounds (Sardans *et al.*, 2009). Ethanol is the volatile compound that is present in higher percentage (up to 8-19% v/v in wine). There are other volatile compounds present in wine, such as methanol or terpenes, but in lower proportions. However, they still influence wine organoleptic properties.

Non-volatile compounds reach concentrations of about 1.0 g L^{-1}, and are represented by sugar, organic acids and conjugated salts. Aminoacids, polyphenols or flavonoids are present in wine, but in much lower concentrations. On the other hand, wine inorganic fraction is rich in Cl^-, PO_4^{3-}, SO_3 and SO_4^{2-}. The ions present in greater quantities are potassium, calcium, sodium and magnesium. Other elements are also present in very low concentrations, for instance selenium, lead and cadmium (Magalhães, 2008; Grindlay *et al.*, 2011).

Minerals present in wine are mostly due to root absorption, and there is a constant enrichment with these compounds, except during fermentation when a decrease of mineral content takes place. During fermentation, the decrease in the concentration of trace elements is due to precipitation of metals as organic salts and/or sulphides, and to absorption by yeasts and bacteria (Grindlay *et al.,* 2011).

Mineral composition has been used as a parameter for quality control and to ensure the wine origin. Wine mineral composition varies during the winemaking process, so it must reflect not only the mineral composition of the soil, but also the changes along the winemaking process. When this fact is not verified, it means either that the wine has been adulterated, or that the grapes to produce the wine are not from the origin that are claimed to be (Catarino *et al.*, 2006).

1.3 Portuguese "terroir"

"Terroir" is a concept that integrates climate, soil, plant material, action, and scientific characterization. In a world of globalization, products of a defined region are valued and seen as a detachment of the "old world" to the "new world", and also as an identity symbol (Catarino *et al.*, 2006; Voica *et al.*, 2009; Andrade, 2010).

Nowadays, “Terroir” is used as marketing in winemaking industry. In Portugal there is only one parcel of 2.5 hectares considered worthy to be designated with this denomination, and has a high status worldwide. This region was born in 1931 and produces the Porto Vintage Quinta do Noval Nacional wine. Also with notoriety, but not in the same scale, are considered Quinta do Crasto Maria Teresa or Mouchão. Along the country there are vineyards or parcels of vineyard in Douro, Dão, Bairrada, Palmela and Alentejo that produce above average in the region, but the wines which result are not consistent enough to win the attribute in the global market (Afonso, 2009). This typology of wine are very expensive, and were not analyzed in this thesis, but in order to avoid the usual confusion between “Terroir” and soil it seemed necessary to explain and briefly characterize both.

1.4 Portuguese wine regions

Already in Roman Empery, it was established that the wines produced in a certain region were better than wines produced in other regions. The first Demarcated Region was delimited in Hungary, in 1700. In Portugal, the first Demarcated Region was defined for Douro, in 1756 (Magalhães, 2008).

Nowadays, Portuguese wines are categorized in three different types: Designation of Origin (DO or DOC), Geographical Indication (IG or IGP) and all the others are designated as table wines (Figure 1.2).

DOC wines are associated to a certain geographic region and have to respect certain rules, such as the soil type where vineyard can be installed, type of castes planted, winemaking process, among other rules. There are 30 regions in Portugal able to produce DOC wines according to Portuguese Ministry of Agriculture, Rural Development and Fisheries (2007) (DECO-Proteste, 2010).

IG wines are produced in specific regions and need to follow certain requisites, such as to have a content of 85% of grapes from a region and be only produced with certain castes. The biggest difference from DOC wines is that the production area is usually wider according to Portuguese Ministry of Agriculture, Rural Development and Fisheries (2007) (DECO-Proteste, 2010).

Currently, 12 regions are defined in Portugal as IG wine producers. These regions are characterized as follows (Tasev *et al.*, 2005; Dominé, 2006).

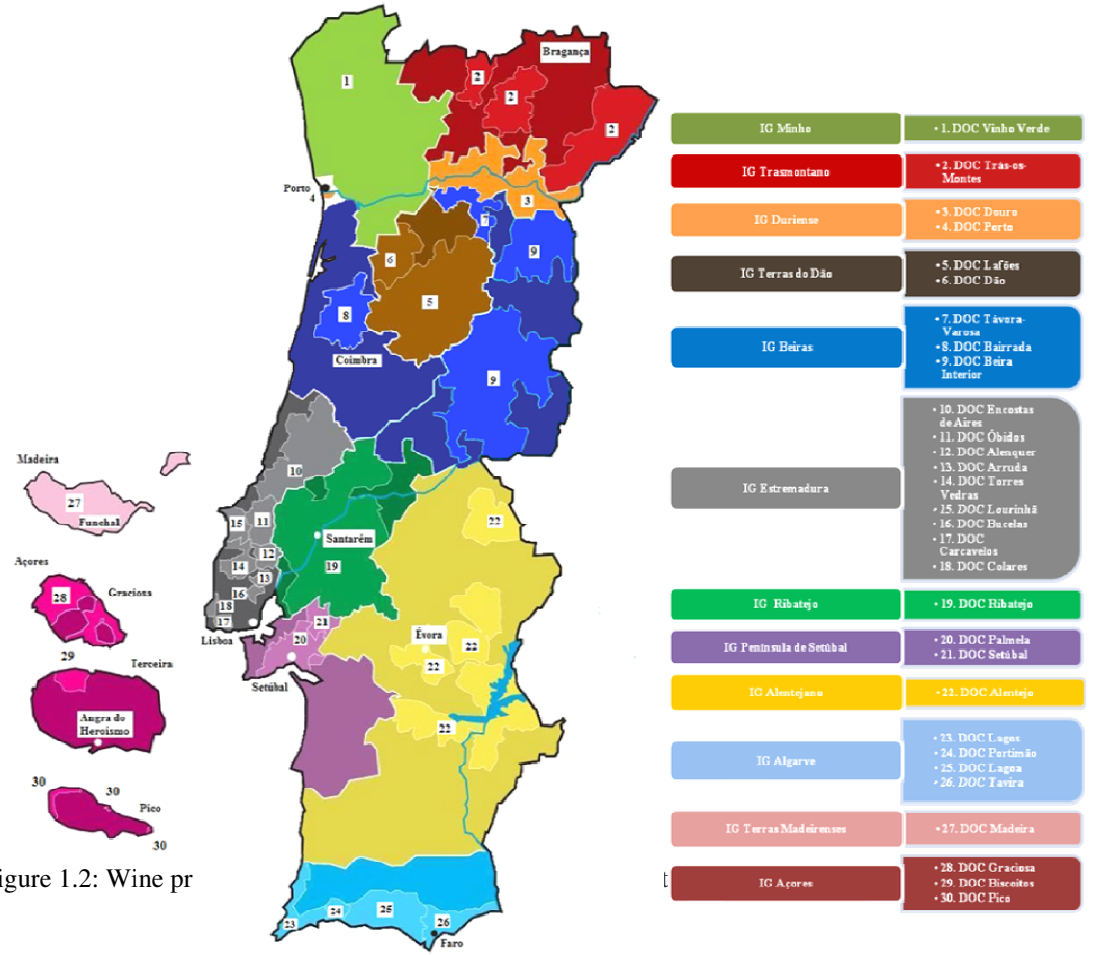

Figure 1.2: Wine pr

- Minho: Region of hills and valleys, with soils, mostly granitic, with soft winters and summers. There is a high level of humidity associated with the sea proximity and the precipitation along the year.

- Trás-os-Montes: Region with mountains and valleys with schist soils. There are different wines associated with this region due to differences on the climate and soil, which led in 2006 to the distinction of Douro region.

- Douro: Region characterized by schist and granitic soils with high slopes. These soils have been worked by men along the years, and represent nowadays a profit for all community.

- Beiras: Region with contrasts between flat coast and hilly interior with high mountains and differentiated climates.

- Dão: Region with schist and granitic soils, with hot summers and rainy winters. The wine aging capacity is of 10-20 years.

- Estremadura: Region with low temperature variation along the year and reduced precipitation. Soils are mainly composed by argil and limestone.

- Ribatejo: Region with moderate temperatures with flat soils that produces different wines, according to the distance of vineyards to Tejo River.

- Terras do Sado: The characteristics of wines from this region are influenced by the proximity to Atlantic Ocean. Two distinct zones can be distinguished; one in altitude with soils of argil and limestone, and the other one located at Ocean level with sandy soils.

- Alentejo: This is a very flat Region of Portugal that produces wines with the perfect conditions for maturation. In the months before grape harvesting, the sun is abundant and the rain is scarce.

- Algarve: In this Region, the soils are composed by argil and schist. The temperature is high and the wind is low, which lead to high alcohol percentages in wine.

- Madeira: Wines from this Region are very typical due to volcanic land and moderate clime.

Açores: This Region has volcanic soils, but more recent than Madeira soils. The climate is characterized by low temperature variations and high precipitation levels (Magalhães, 2008; Stafilov & Karadjova, 2009).

1.5 Trace elements and toxic effects

Trace elements have different definitions in science, but there are some common factors to them: a) trace elements are metals and non-metals that have relatively high density; b) they are associated with pollution; b) some of them imply toxicity for consumers (Catarino *et al.*, 2006; Duruibe *et al.*, 2007). Nevertheless, some of these elements are needed in small concentrations for human body, such as Ni. On the other hand, elements like Pb, Cd, As and Hg are potentially toxic even in small amounts (Garrido *et al.*, 1997; Stafilov & Kardjova, 2009; EFSA, 2009).

Some trace elements are constituents of herbicides and pesticides used for vineyard treatment (Tasev *et al.*, 2005), but the accumulation in grapes and wine also comes from environmental pollution, equipment used in winery and methodologies performed from vineyard to bottling (Dessuy *et al.*, 2007; Voica *et al.*, 2009; Fiket *et al.*, 2010; Moreira *et al.*, 2011). Pesticides and herbicides used in soil treatment can be accumulated in skin grape or be absorbed by the root of the vine (Orescanin *et al.*, 2003; Sardans *et al.*, 2009). During wine production, the equipment composition with metallic alloys can contribute to increase the content of trace elements in the final product, as well as the recipients where wines are aged and glass bottles where wines are stored and sold (Pera *et al.*, 2008; Fiket *et al.*, 2010).

The toxicity effects of trace elements are well studied, and the major contributors for the accumulation in human body are food, water, and beverages (Karadjova *et al.*, 2006).

Trace elements are minority compounds that not only affect the organoleptic perception of wine, but also influence positively or negatively the consumer health (Rivero-Pérez *et al.*, 2008; Garrido *et al.*, 1997).

It has been proved that the consumption of wine, especially red wine, provides essential minerals to dietary intakes, and also antioxidants that are important to eliminate the effect of free radicals (Stafilov & Karadjova 2009; Burin *et al.*, 2010; Bukovan *et al.* 2010). On the other hand, there are trace elements that can be toxic and even carcinogenic for consumers when ingested in large amounts (Garrido *et al.*, 1997). Trace elements are present in wine naturally, because they are part of environment. But, there are external sources of contamination, like environmental pollution, herbicides and pesticides (Tasev *et al.*, 2005; Fiket *et al.*, 2010;

Moreira *et al.*, 2011; Donadini *et al.*, 2008) that can interfere with wine organoleptic features (Bukocvan *et al.*, 2009). Ni can interfere with wine properties, because of haze formation that gives an undesirable taste and flavor, due to the tendency to form complexes with tannins and anthocyanins (Tariba, 2011). In terms of human health, trace elements, such as Cd and As, do not have any function in human body, and are considered unnecessary in any concentration in terms of Recommended Daily Intakes (RDI) (Stafilov & Karadjova, 2009; Tariba, 2011).

Arsenic is not needed for human health in any quantity and must be carefully quantified in wine as it is currently present in low concentrations (Garrido *et al.*, 1997; Stafilov & Karadjova, 2009). Its bioavailability in human body depends in large scale of the physico-chemical species in which As is in the compound ingested and how the body metabolizes it. Intoxications with As range from not very harmful up to of being associated to different kinds of cancer, especially skin cancer (Mandal & Suzuki, 2001; Donadini *et al.*, 2008). The toxicity decreasing scale of As chemical species is as follows: As(III) > As(V) > monomethylarsonic acid (MMAA) > dimethylarsinic acid (DMAA) (Catarino *et al.*, 2008). These As chemical species exist naturally, being the inorganic forms more toxic than the organic ones (Herce-Pagliai *et al.*, 2002). The study of the total content of As in food is nevertheless essential, despite the chemical form in which As is present in it, because the legislation does not differentiate among the different chemical forms of As (Sardans *et al.*, 2009).

Pb contamination is an ancient story, Romans used Pb vessels to store wine, and Pb acetate sweeteners were added to wine for color improvement and preservation. It seems that these practices in processing the “drink of Gods” were one of the reasons for the decline of the Roman Empire. Pb is a trace element of major concern, because of its high toxicity and presence in the equipment used to produce bottled wine (Green & Scollary, 2000; Palacios *et al.*, 2001). Pb concentration found in wine is nowadays in lesser concentrations than the stipulated by legislation (Sanz *et al.*, 1989; Catarino *et al.*, 2009; Voica *et al.*, 2009).

In wines of Hungary and Serbia, Cd was found in large concentrations: in the first case it was due to a contamination during the production of vineyard; in the second case it was because of the proximity of the winery to a traffic zone (Tariba, 2011). Cd is carcinogenic and can be introduced into the body through airway, ingestion and skin contact. Usually it is present in the form of sulphide and is strongly associated with the ores of zinc. Afterwards, it is absorbed by the roots of the vine (Catarino *et al.*, 2009).

Hg is a very toxic element, and its bioavailability depends on the chemical form; inorganic salts are usually less toxic than organomercury chemical species. Hg is stored in sediments and is introduced in the food chain, due to microbial activity which turns metallic Hg into organic Hg,

in the form of methyl-mercury, which is extremely volatile and toxic (Karadjova *et al.*, 2005; Catarino *et al.*, 2009).

Ni is necessary for normal function of human body up to certain concentrations, but in higher concentrations is toxic (Cempel & Nikel, 2006). It is necessary in human body in the quantity of 1 mg kg^{-1} per day for adults, according to the National Academy of Sciences (2006), but above this value this element turns toxic. In the last years, it might have been a slight increase in the content of Ni during the production process of wine, due to the materials used to produce equipments which contain Ni (Catarino *et al.*, 2006).

Trace elements trigger different effects on human body, depending on the element and the chemical species in which they are present in wine (Table 1.2). But, there are common effects, such as gastrointestinal disorders, tremor, paralysis, vomiting and convulsions and even depression (Table 1.2) (Mandal & Suzuki, 2001; Karadjova *et al.,* 2006).

Table 1.2: Principal sources of contamination by trace elements in wine and associated effects (Catarino *et al.*, 2006; EPA, 2011).

Trace element	**Contamination source**	**Impaired organ or system/Effect**
As	Pesticides and herbicides (presence of arsenious salts)	Gastrointestinal tract; central nervous system; cardiovascular system; associated to various cancers
Hg	Environmental contamination	Gastrointestinal tract; nervous system and kidneys
Cd	Environmental pollution (contact with materials with Zn, always reach in Cd)	Pulmonary irritation; severe kidney problems
Pb	Environmental pollution; cupric fungicides (Pb is an impurity is always present); materials containing Pb alloys; crystal bottles	Nervous system; malformation of fetus
Ni	Environmental pollution; stainless steel; bottling conservation (due to pigments with Ni)	Damage to the lungs and kidneys; gastrointestinal distress

Several trace elements, such as Ni, Pb and Hg, are not considered carcinogenic by International Agency for Research on Cancer (IARC), while others revealed to be carcinogenic to humans, such as for example, As and Cd (Schwartz, 1975; Boffetta, 1993). Arsenic revealed to increase the skin cancer, and Cd enhanced the risk of lung cancer (Catarino *et al.*, 2008; Donadini *et al.*, 2008).

In order to evaluate the danger for human health, not only based on the maximum levels defined by several organizations, like the Organisation Internationale de la Vigne et du Vin, it was developed an equation to measure the risk for human health, which was named as Target Hazard Quotients (THQ) (Herce-Pagliai *et al.*, 2002; Sardans *et al.*, 2009).

THQ is a dimensionless parameter that relates the concentration of an inorganic compound to the amount of exposure along the time, the carcinogenic capacity of the element and the body weight (Grindlay *et al.* 2011). It is considered safe for public health a value of THQ below 1. Values above 1 are considered a reason for health concern (Hague *et al.*, 2008; Naughton & Petróczi, 2008).

Red wines from all over Europe revealed potentially high concentrations of trace elements by a study developed in Kingston University (Naughton & Petroczi, 2008). The aim of this study was to calculate THQs in 15 different countries of Europe, South America and Middle East. France, Spain and Portugal revealed values above 100. It was found values above 350 for Hungary and Slovakia, and there is a broad list of countries with values above 1(France for instance)

THQ is calculated according to equation (1.1) developed by the Environmental Protection Agency (EPA):

$$THQ = \frac{EFr \times EDtot \times SFI \times MCSinorg}{RfD \times BWa \times ATn/c} \times 10^{-3} \qquad (1.1)$$

Where EF_r is the exposure frequency (days year^{-1}) for both men and women, ED_{tot} is the exposure duration (year), *SFI* is the mass of the selected dietary ingested (g.day^{-1}), MCS_{inorg} is the concentration of inorganic species in study, which in this case is the concentration of the trace elements that were quantified in the wine samples studied in this thesis (µg g^{-1}), *RfD* is the oral reference dose (mg kg^{-1} day^{-1}), BW_a is the body weight for both men and women (kg) and $AT_{n/c}$ is the average time (days) for non-carcinogenic trace elements (AT_n) or for carcinogenic trace elements (AT_c) (days) (Hague *et al.*, 2008; Naughton & Petróczi, 2008).

1.6 Atomic Absorption Spectrometry

Trace elements analysis and quantification in food and beverage samples are often determined with Atomic Absorption Spectrometry (AAS) techniques (Buldini *et al.*, 1999). Because different elements are present in different concentrations for the same matrix, there is an appropriate technique for each case. AAS analysis can be performed by Hydride Generation Atomic Absorption Spectrometry (HGAAS), Electrothermal Atomic Absorption Spectrometry (ETAAS), or Flame Atomic Absorption Spectrometry (FAAS). For each technique there are different quantification sensitivities, methods of atomization or maximum reached temperatures, and when choosing the right one for the trace element quantification all these parameters must be taking into account.

Each element has a number of electrons associated with the nucleus, and the most stable configuration for an orbital of an atom is called "ground state". The atoms in "ground state" absorb light of a characteristic wavelength and passes to the "excited state". When more atoms go into an "excited state" more light is absorbed. With the measurement of the light absorbed it is possible to determine the analyte concentration in the sample in study. The broader is the wavelength emitted by the light source the highest is the quantification accuracy of the element in study (Skoog *et al.*, 1997).

The atomic absorption instrumentation is composed by various crucial elements:

- Light source: emits a specific wavelength correspondent to the analyte (hollow cathode lamp, for example).
- Monochromator: provides light dispersion to isolate the correct wavelength.
- Detector: measures the light and amplifies the signal.
- Readout instrument: shows the reading after being processed.

The measurements with AAS are based on Beer–Lambert law, that relates the absorption with the analyte concentration ($A= \varepsilon bc$).This must be a linear correlation or the law is not respected and therefore the concentrations determined are not accurate. The molar absorbtivity is represented by ε ($L\ mol^{-1}\ cm^{-1}$), b is the path length of the cell (cm) and c is the analyte concentration ($mol\ L^{-1}$) (Perkin-Elmer, 1996).

Despite FAAS, HGAAS and ETAAS being based on the same basic principles that were described above, there are some differences between techniques. In FAAS the liquid sample is aspirated, aerosolized and mixed with combustible gases (air-acetylene for instance).

Afterwards, the mixture is exposed to a flame that has a temperature ranging from 2100 to 2800°C. During the combustion, the atoms of the analyte are excited and absorb light in the characteristic wavelength (Skoog *et al.*, 1997).

HGAAS is based on the hydride generation of the element in study, which is volatile. For Hg, the technique does not require flame, because it is based on the volatility of the Hg in the ground state at room temperature. This element is reduced to a free atomic state with a reaction with a strong reducer agent ($NaBH_4$ for instance) that follows the chemical reaction (1.1). There are some matrixes that require a pre-reducer agent like potassium iodide (KI) to enssure that the correct physico-chemical form for the quantification is produced (Wifladt *et al.*, 1996; Karadjova *et al.*, 2005).

$$Hg^{2+} + 2BH_4^- \leftrightarrow Hg^0 + B_2H_6 \quad (1.1)$$

After the formation of the volatile Hg, this is transported by a gaseous flow to the radiation path in the spectrometer.

Also with HGAAS, but with flame heating is performed the quantification of As. The technique is very similar to that of Hg, but instead of producing free atoms it produces volatile hydrides, and those hydrides are dissociated by the flame in free atoms. The protons from the $NaBH_4$ react with the element in study, as shown in chemical reactions 1.2 to 1.4.

$$M_3 + 3H^{\cdot} \leftrightarrow MH_3(volatile) \quad (1.2)$$

$$M^{2+} + 2H^{\cdot} \leftrightarrow MH_2(volatile) \quad (1.3)$$

$$MH_2 + heat \leftrightarrow M^0 + H_2 \quad (1.4)$$

HGAAS is a very interesting technique due to its low detection limits, but it is only applicable for a few elements because of the incapacity of some compounds to form a volatile hydride (Skoog *et al.*, 1997; Harris, 2002).

ETAAS is a technique that does not use flame for the atoms formation. Instead it uses an electrothermal heating produced in a graphite tube. In the graphite tube the heating program is developed until the atomization of the analyte occurs. The heating program is composed by three steps: 1) drying; 2) pyrolisis; 3) atomization. Drying step is performed under soft temperatures (100ºC - 120ºC) and the solvent is eliminated. In the pyrolisis step, the organic and

inorganic matrix compounds are volatilized. In the atomization step, it is formed the atomic vapor composed by the analyte. ETAAS is very sensible to matrix interferences, which can be minimized by the presence of modifiers. Modifiers allow the analyte to be quantified with less interference, either by stabilizing the analyte or by reacting with the interfering compounds leaving the analyte free for quantification (Skoog *et al.*, 1997; Harris, 2002).

1.7 Analytical determination of trace elements in wines

In the last years, the analytical determination of trace elements in wines has been improved. About 40% of the analyses reported in literature are performed with Inductively Coupled Plasma Mass Spectroscopy (ICP-MS); 16% with ETAAS; 15% with FAAS; less than 7% with HGAAS (Table 1.3). To choose the most accurate technique, it must be taken into account the following factors: trace element concentration, number of trace elements in study and possible interferences.

Table 1.3: Methodologies described in literature for each trace element is study.

Trace element	Methodologies used	References
As	HGAAS; ETAAS; ICP-MS	Karadjova *et al.*, 2005; Tasev *et al.*, 2005; Husáková *et al.*, 2007
Hg	HGAAS; ICP-MS	Karadjova *et al.*, 2004; Sardans *et al.*, 2009; Fiket *et al.*, 2010
Cd	ETAAS; ICP-MS; FAAS	Stozhco *et al.*, 2007; Korn *et al.*, 2008; Sardans *et al.*, 2009
Ni	ETAAS; FAAS; ICP-MS	Thiel *et al.*, 2004; Burin *et al.*, 2010; Fiket *et al.*, 2010
Pb	ETAAS; FAAS; ICP-MS	Sanz *et al.*, 1989; Dessuy *et al.*, 2007; Karadjova *et al.*, 2006; Elçi *et al.*, 2009; Sardans *et al.*, 2009

If the aim of the work is the determination of several elements in a broad concentration range at the same time, ICP is usually used. The analysis can be improved, namely the detection limits, through a sample pre-preparation that can comprise for instance an acid digestion.

Elements present in high concentrations, like Ca and Mg, are usually determined by FAAS. This technique has been used for trace elements quantification by OIV and EU. If the elements are present in low concentrations, it is also possible to quantify by FAAS if a previous step of pre-concentration is performed.

When the trace element is present in a very low concentration, ETAAS, HGAAS or ICP-MS techniques are currently used. ETAAS is an interesting approach for this determination, because the matrix interference is suppressed by the pirolysis step and the addition of the matrix modifier. HGAAS on the other hand ensure lower detection limits than FAAS and it is less time consuming than ETAAS (Grindlay *et al.*, 2011).

1.7.1 Matrix interference

Matrix interference can either enhance or diminish the signal of the analyte. Most of the reports in literature make reference to matrix interferences and describe methodologies to diminish them. These interferences represent a distortion in the detection limits, as well as in the accuracy or precision of the technique used (Grindlay *et al.*, 2011).

Ethanol is the volatile compound present in higher concentrations and introduces spectral interferences in the quantification, due to background noise. For FAAS this problem is of low significance, because of the flame strength and the fact that with this technique it is possible to use high volume of solvents.

HGAAS is affected in the generation of volatile compounds, which lead to vapor moved into the atomizer, variation of the pH and the oxidation capacity of the elements in reaction.

With ETAAS, the choice of matrix modifier is crucial for an accurate determination, but attention should be kept to the fact that some of the organic non-volatile compounds can act as modifiers. On the other hand, the incomplete pyrolisis can accumulate wastes and produce smokes that with time will affect the measurements (Grindlay *et al.*, 2011).

1.7.2 Suppression of matrix effects

A broad range of methods are used for the suppression of several matrix effects, in order to enhance the precision and accuracy of trace elements' quantification, varying from general methods applicable to the majority of techniques (dilution for instance) to more specific ones for each technique (use of modifiers for ETAAS for instance) (Fernandes *et al.*, 2001).

Sample pre-preparation has been improved in the last years in order to be less time consuming and more cost effective. This very important step on the quantification of trace elements in

wines may include dilution, acidification, evaporation or digestion (Misiego *et al.*, 2004; Pera *et al.*, 2008; Korn *et al.*, 2008).

The dilution does not require a lot of steps. Therefore, it will not affect in great extent the final result. But it has to be chosen the correct dilution factor depending on the concentration of the analyte in wine and the final concentration that is intended to be reached. With the increase of dilution there is a suppression of the matrix effects. On the other hand, if the trace elements are present in wine in low concentrations, the quantification may be difficult. It can also influence wine pH value, which lead to stability modification; salts like K and Ca are not soluble in high pH values, so they will precipitate if acid is not added to wine solution during dilution.

Alcohol removal is accomplished by a simple evaporation that can be performed by heating or using a rotary evaporator. Once the alcohol is removed, the initial sample volume is completed with ultra-pure water.

Acidification of wine samples is usually performed to assist the atomization during quantification, and also to oxidize organic matter making free the trace elements for quantification.

To reduce the wine matrix interference by organic compounds decomposition, digestion methods can be used, for instance microwave or digiprep systems. Usually these techniques are based on sample heating in the presence of HNO_3, HNO_3+HCl and eventually H_2O_2. There are some disadvantages associated to this technique, namely high volume of reagents, possible loss of volatile compounds or contamination of wine samples due to reagent contamination (Grindlay *et al.*, 2011).

1.6 Maximum levels

Although legislation defines for each country what is allowed in terms of winemaking process (Table 1.4), its effective application depends on what each region considers more correct for wine production (Patti *et al.*, 2009). Legislation all over the world defines the quantities of various elements in wines, including trace elements. If the legislation is not fulfilled, two different facts can occur: either there is a break of the law without problems for human body, or a break of the law lead to a concern for public health (Catarino *et al.*, 2006).

Table 1.4: Maximum levels defined by OIV and European Community (Regulation (EC) Nº 1881/2006) concerning the five trace elements considered in the present thesis.

Species	Maximum levels by OIV (μgL^{-1})	Maximum levels by European Community (μgL^{-1})
As	200	ND
Cd	10	ND
Pb	150	200
Hg	ND	ND
Ní	ND	ND

ND: not defined.

The maximum concentration defined by the Organisation Internationale de la Vigne et du Vin for As in wines is of 200 $\mu g\ L^{-1}$, but the concentrations in literature are usually below this value (0.5-30 μgL^{-1}) (Moreira *et al.*, 2011).

Cd concentration has a limit value defined by OIV of 10 μgL^{-1}. Usually the wine content in Cd is less than 5 μgL^{-1} (Tariba, 2011).

Pb has a limit value established by OIV at 150 $\mu g\ L^{-1}$ (Azenha & Vasconcelos, 2000). The mean values found in wines described in literature are of 17 μgL^{-1} (Sanz *et al.*, 1989; Catarino *et al.*, 2009; Voica *et al.*, 2009). The only restriction applied in Portugal by law for trace element levels in wines is for Pb and it is defined at 200 μgL^{-1} in the Regulation (CE) nº 1881/2006.

OIV does not have maximum levels defined for Hg and Ni. Therefore, in this work Ni is compared with the Tolerable Upper Intake Levels (defined by The National Academy Press, 2006) and Hg compared with Provisional Tolerable Weekly Intake (PTWI defined by FAO/WHO, 2010). Ni is necessary for normal function of human body up to 1 mg kg^{-1} day^{-1}, in higher concentrations can be associated to toxic effects. Hg is tolerable up to 1.6 $\mu g\ kg^{-1}$ $week^{-1}$ for an adult, but in higher concentrations is toxic (Cempel & Nikel, 2006).

1.8 Justification of thesis subject selection

As more people and more countries are interested in wine, this drink takes a leading role and is responsible for new experiences, from vintage to tasting courses. The time in which wine was exclusive to elites has passed on and, nowadays, most of the wines are available to everyone.

In this context, the wine should be subjected to safety and quality controls as stringent as any other type of food. When a bottle of wine is bought, the only descriptions that are in the label are the alcohol content, the varieties of castes that were in their origin and the place of

production. If the consumer wishes to known the information on the composition of the wine, this is not available to the public. However, worldwide there is a wide range of different search features in this regard, especially in the quantification of trace elements (Karadjova *et al.*, 2005; Dessuy *et al.*, 2007; Sardans *et al.*, 2009; Burin *et al.*, 2010).

Trace elements differ from other toxic agents, because they are not synthesized or destroyed by human organism. The presence of these metals in wines is due to their presence in water, soil, and pesticides used in disease prevention or treatment of the vines (Nasir *et al.*, 2001). The forms to control the metal content in wines can go from restricting the use of phyto-pharmaceutical products to ban the production of vineyards in contaminated soils. In high amounts, poisoning with trace elements can cause acute or chronic effects, including damages in central nervous system, reduction of mental function, changes in blood composition, and changes in the functions of vital organs such as lungs, kidneys and liver. If the exposure is extended, there may be neurological degenerative processes which mimetize symptoms of Alzheimer's, Parkinson's or multiple sclerosis.

There is not a significant number of published studies on the concentration of trace elements in Portuguese wines delivered for consumers. Validation studies of analytical techniques for the quantification of heavy metals in Portugues wines are more current, such as the study of Neves (2010). There are also assessments for the presence of Pb and Cu in wines through speciation techniques (Azenha and Vasconcelos, 2000), measurements of elements considered as wine contaminants (Catarino *et al.*, 2006), quantitative determination of heavy metals in different stages of wine production (Pessanha *et al.*, 2010) and also a study in the wines of Madeira and the Açores to the level of metal ion content (Trujillo *et al.*, 2009).

Due to the fact that Portugal is a producer of relevance in the world scene, presenting a high percentage of wine exportation, it is imperative the development of a study to quantify trace elements in some marketed wines. This thesis pretends to be a contribution for that study.

This thesis has a differentiation factor from the research studies presented earlier: in this thesis a comparative study between different national wine producing regions with Geographical Indication (IG) was performed.

1.9 Aims of the thesis

This thesis aims to study the presence of five trace elements (As, Cd, Hg, Pb and Ni) in Portuguese red wines available in the market. The choice of conducting the study only in red

wines was due to the fact that this is the wine type with the highest production and the most consumed in Portugal.

In the first part of the thesis, it was necessary to understand in what extent the Portuguese red wines needed a sample pre-preparation to perform an accurate quantification of the trace elements. Associated with this step of the study it was the definition of the reagents and respective concentrations in which they were necessary to enable the quantification of trace elements. Also in this part of the study it was determined the relevance of acid digestion for the quantification. This part of the experimental work was named as Method Validation. Afterwards, 25 Portuguese red wines and their duplicates were submitted to the quantification of five trace elements. The concentrations determined were compared with the European legislation.

In the second part of the thesis, a statistical analysis of the data obtained was performed. In this analysis, the Portuguese wine producing regions with the highest concentrations of the five trace elements were determined. It was also possible to determine which trace elements explain better the system variance, being the system composed by the trace elements and Portuguese wine producing regions.

In the final part of the thesis, THQ values were determined in order to assess the level of concern to public health that can be associated to each wine, producing region and consumer gender. This is a different approach from the first analysis, due to the fact that it is not a comparison of concentrations with limit values, but it is a study that allows understanding whether the drinking of Portuguese red wines that were studied can be a motive of concern for public health.

2. Experimental

2.1 Wine samples

Although there are in Portugal 12 regions with IG denomination, Açores does not produce red wines. Therefore, the study only covered 11 national wine regions. The selection of wines were made by the 11 IG regions that produce red wines, with the number of selected wines according to the average volume of red wine produced in these regions. The total number of studied wines was of 25 brands, plus their duplicates. The wines were purchased in local supermarkets at a range cost of 4 to 5 Euros/liter. This range price provides a very comprehensive choice of brands, with a satisfactory quality/price ratio, and with this price it is possible for the producer to enter in detail in the vineyard and winery and obtain a quality wine (Coutinho, 2011). The selected red wines were from the last crop year (2009) before this study had been started, bottled in glass containers with a capacity of about 1 liter and cork sealed.

Six different red wines with duplicates, from different regions of Portugal, were analyzed for method validation. The wines analyzed in method validation were multicast and came from the regions of Alentejo, Estremadura, Douro and Terras do Sado. All were classified as Geographical Indication (IG), which allows the consumer to know that these beverages have guarantee of origin and quality certificate.

For the determination of trace elements in wines representing the country as a whole, the wines had also the classification of Geographical Indication (IG). In this step of the work, a total of 25 multicast wines and their duplicates that covered all the eleven Portuguese red wine producing regions were analyzed.

For the statistical analysis, only seven Portuguese red wine producing regions were considered, which corresponded to the regions with the higher production of red wine in the country: Alentejo, Douro, Beiras, Minho, Terras do Sado, Estremadura and Ribatejo. According to Portuguese National Institute of Statistics (INE, 2008), Alentejo is the highest contributor for national production with a value above 300.000 hL per year; Ribatejo is the wine producing region that contributes with less wine, with a volume of 47.500 hL per year.

2.2 Sample pre-treatment

Sample pre-treatment is an important step to enable the elements for an accurate quantification. Often the trace elements are trapped in the wine matrix or complexed with other wine

components, such as pigments. If the treatment prior to the quantification is not efficient, it is very probable that the total quantification of a certain element is not being performed adequately. Table 2.1 summarizes all the treatments used for As, Hg, Cd, Ni and Pb quantifications which are explained afterwards.

Table 2.1:Pre-treatments performed for trace elements method validation.

Trace element	Sample pre-treatment	AAS techniques
Cd	Addition of spike	ETAAS
	Absence of spike	
	Acid digestion	
	Acidification without digestion	FAAS
	Addition of H_2O_2	
	Absence of H_2O_2	
Ni	Addition of spike	FAAS
	Absence of spike	
	Acid digestion	
	Acidification without digestion	
	Addition of H_2O_2	
	Absence of H_2O_2	
Pb	Addition of spike	ETAAS
	Absence of spike	
	Acid digestion	
	Acidification without digestion	FAAS
	Addition of H_2O_2	
	Absence of H_2O_2	
Hg	Addition of spike	HGAAS
	Absence of spike	
	Acid digestion	
	Acidification without digestion	
	Addition of H_2O_2	
	Absence of H_2O_2	
As	Addition of spike	HGAAS
	Absence of spike	
	Acid digestion	
	Acidification without digestion	
	Addition of KI	
	Evaporation in water bath	

All samples were acidified with 3 ml of 0.11M HNO_3 for ETAAS, 1.1M HNO_3 for FAAS, 0.65M HCl for Hg and 3.24M HCl for As by HGAAS. This acidification pre-treatment was

performed to liberate the trace elements from the matrix, to enable their quantification, and to facilitate the atomization process.

There are a number of reported interferences during the analysis of trace elements. For example, ethanol interferes in the quantification of trace metals during sample atomization, and these metals can be unavailable for quantification because of complexation with some elements of the matrix, such as pigments (Stafilov & Karadjova, 2009). Because of these difficulties, the effect of an acidic digestion and controlled temperature was firstly studied. In a second step, the influence of the addition of hydrogen peroxide on trace metals quantification was assessed for Hg, Cd, Pb and Ni. For As quantification, several sample preparations were studied: a) direct analysis; b) acid digestion; c) water bath evaporation. Besides those techniques, the influence of potassium iodide in As reduction was also studied.

Generally, the literature reports low concentrations of trace elements in wine matrices (Lara *et. al.*, 2005; Voica *et al.*, 2009). Therefore, all wine samples were analyzed with and without spike. For Hg and As, quantified by HGAAS, the wines were spiked with a standard solution for a concentration of 1.25 μgL^{-1}; for As quantification by HGAAS, the concentrations of spike achieved a value of 1.5 μgL^{-1} for all pre-treatments, except for evaporation where the spike concentration was of 0.37 μgL^{-1}; for Cd and Pb, quantified by ETAAS, adequate volumes of standard solutions were added to achieve a concentration of 1 mgL^{-1}; for Pb, Cd and Ni, quantified by FAAS, the wines were spiked with proper volumes of standard solutions for an initial concentration of about 100 mgL^{-1}.

2.2.1 Acid digestion

"Digiprep" was the method chosen to subject the samples to a pre-acidic digestion under higher and controlled temperature. This system performs a soft digestion, because it is done under atmospheric pressure at low temperatures (≤100ºC). It was not necessary to perform a more aggressive acidic digestion, such as microwave digestion, because wine matrix is not highly complex in terms of organic compounds. The digestion minimizes the matrix effects (e.g. ethanol) that can interfere with the quantification of the total content of metals.

To perform the "Digiprep" digestion, 15ml of wine was placed in PTFE vessels, with 3 ml of H_2O_2 and 3 ml of 0.11M HNO_3 for ETAAS, and 1.1M HNO_3 for FAAS. For the HGAAS, it was used the same amount of wine and H_2O_2, but instead of HNO_3 it was added 3 ml 0.65M HCl for Hg and 3.24M HCl for As. The program selected for the digestion was as follows: 15 min ramp, up to 40ºC, and a platform of 1 min under this temperature; 5 min ramp, up to 100ºC, and a platform of 60 min under this temperature. After the digestion process and cooling down

to 30ºC, the sample volumes were corrected to 50 ml with ultra-pure water (Milli-Q Academic from Millipore).

In the case of wine samples that were not submitted to this previous digestion process, equal amounts of H_2O_2, HNO_3 and HCl were added to wines and the final volume were corrected to 50 ml, but no heating process was performed.

2.2.2 Hydrogen peroxide

This reagent can be used to oxidize the organic matter of wines, making the trace elements free for quantification. Wines have different compositions all over the world. Therefore, it was necessary to understand if hydrogen peroxide was needed to perform an accurate analysis in the case of Portuguese red wines. For this purpose, all wine samples were analyzed with 3ml of H_2O_2 (0.8M), and also without the addition of peroxide hydrogen as control assay.

2.2.3 Evaporation in water bath

During the atomization step, ethanol is one of the interferences most described in literature. To avoid this problem, a simple technique was tested for its loss: evaporation in water bath, at 40ºC, in open PTFE vessels. In the spiked samples, a proper volume of 0.75 $\mu gAsL^{-1}$ was added for a final concentration of 0.0125μgL^{-1}. Wine samples were evaporated to half of the initial volume, which was of 30 mL. After evaporation, the volume of wine samples was corrected to 30 mL with ultra-pure water.

2.2.4 Reduction of As by potassium iodide

Potassium iodide was added to wine solutions to reduce As forms to As (III), in order to make As available for quantification by HGAAS. The mixture between sodium borohydride and sodium hydroxide is not enough to reduce As and As compounds to As (III) (Karadjova *et al*., 2005). To promote this reduction, 0.5 g of KI was added to non-digested and non-spiked wine samples.

2.2.5 Instrumentation

DigiPREP MS with a coupled DigiVac system from SCP SCIENCE was used for acidic digestion. This equipment digests 48 samples at once.

The quantification of Hg and As was performed by HGAAS in a Unicam SOLLAR M Series Flame Absorption Spectrometer with VP90 Continuous Flow Vapor Accessory. The analysis followed the specifications of the spectrometer software.

FAAS was used for the quantification of Cd, Pb and Ni. This was performed by using a ZEEnit 700 atomic absorption spectrometer from AnalyticJena. The Zeeman graphite tube furnace

coupled to the spectrometer was used to quantify Cd and Pb. The selected methodology was based on the apparatus instructions for each trace element.

The hollow cathode lamps used on all apparatus to determine Hg, Cd, Pb and Ni were from UNICAMP analytical system.

The water bath used to perform the evaporation of wine samples was from Memmert.

2.3 Reagents

The solutions were prepared with ultra-pure water from Milli-Q Academic. All material used was decontaminated for 24 hours in 2.2M HNO_3 followed by 24 hours in ultra-pure water.

All standard solutions were prepared by using stock solutions (from Merck) of 1000 mg L^{-1} and HNO_3 0.5 M, except for Hg and As which were prepared by using HNO_3 2 M. HCl (12M) and HNO_3 (16M) for solutions concerning ETAAS and FAAS quantification techniques were from Panreac. KCl needed to prepare standard solutions for FAAS was obtained from Panreac. The hydrogen peroxide used to perform oxidation of the organic matter was also from Panreac.

Sodium hydroxide used for HGAAS determination was obtained from Merck. Sodium borohydride was from Fluka analytical. The modifier needed to perform ETAAS was ammonium phosphate which was from Fluka analytical. The final solution of 4.3×10^{-3}g mol^{-1} $NH_4H_2PO_4$ was diluted in ultra-pure water.

2.4 Quantification of trace elements

All wines submitted and not submitted to the pre-digesting process were analyzed with and without spike. Hg and As were quantified by HGAAS, Pb and Cd were analyzed by ETAAS and FAAS, and Ni was determined by FAAS.

The range chosen to establish de calibration curve for Hg and As by HGAAS was equally distributed from 1.5 μgL^{-1} up to 10 μgL^{-1} for Hg and from 0.5 μgL^{-1} up to 10 μgL^{-1} for As.

The apparatus used for ETAAS technique makes automatic dilutions to trace the calibration curve. In this case, 0.11M HNO_3 was used. Therefore, it was only necessary to define solution concentration through the equipment software, and the different concentrations needed to establish the calibration curve. The standard solutions initially added in the apparatus had concentrations of 40 μgL^{-1} for Pb and 15 μgL^{-1} for Cd.

Pb, Cd and Ni standard solutions were quantified by FAAS using 0.22M HNO_3 and 0.013 g mol^{-1} KCl. The calibration curve for Pb ranged from 1 mgL^{-1} up to 28 mgL^{-1}; for Ni, the lowest concentration was of 0.5 mgL^{-1} and the highest concentration of 6 mgL^{-1}; for Cd, the calibration curve was from 0.5 mgL^{-1} up to 10 mgL^{-1}.

2.5 Statistic analyses

"STATISTICA 8" from Statsoft was used to perform the statistic analyses in this study.

Principal Component Analysis (PCA) was used to determine the variables that better explained the total variance of the system. The statistical system was composed by the trace elements quantified in this study and the seven Portuguese red wine producing regions considered in the statistic analyses.

One-way ANOVA was performed in order to understand how the trace elements were distributed in the studied regions.

2.6 THQ calculation

The Target Hazard Quotients (THQ) were calculated according to the equation (1.1) shown in the "Introduction" chapter.

In equation (1.1), EF_r was considered to be of 14.37 days $year^{-1}$ for both men and women, and *SFI* of 1.96 drinks per day, being a drink equal to a volume of 250 mL of red wine. Both EF_r and *SFI* were obtained from the Eurobarometer of the European Comission (2003).

ED_{tot} was considered to be different for women and men as the life longevity is also different for both genders; 56 years was considered for men and 62.6 years for women, with 18 years old being the starting age of drinking (already discounted in the values presented above), according to the Organisation for Economic Cooperation and Development (OECD, 2004). This value can be conservative since it has been noticed that teen people are starting earlier the consumption of alcohol beverages. Nevertheless, the consumption pattern in the starting years of consumption is highly focused in beers and distillated beverages than in wine.

MCS_{inorg} assumed the concentrations of the trace elements determined in the 25 red wine samples studied in this work. The concentrations expressed in μgL^{-1} were converted to $\mu g\ g^{-1}$ of wine based on a volumic mass of 0.98 kgL^{-1} of red wine (Birse, 2007).

RfD was established for each trace element as follows: As = 0.3×10^{-3} mg kg^{-1} day^{-1}; Hg = 0.1×10^{-3} mg kg^{-1} day^{-1}; Cd = 1×10^{-3} mg kg^{-1} day^{-1}; Ni = 2×10^{-2} mg kg^{-1} day^{-1}; Pb = 1.5 mg kg^{-1} day^{-1}, according to United States Environmental Protection Agency (EPA, 2011).

BWa was considered to have different values for each gender. This parameter was fixed in 63.5 kg for Portuguese women and in 75.9 kg for Portuguese men. These weight values were obtained from the Portuguese Society of Hypertension (2006).

The averaging time for non-carcinogenic (AT_n) trace elements (Ni, Pb and Hg) was calculated by multiplying the ED_{tot} for 365 days. The averaging time for carcinogenic (AT_c) trace elements (As and Cd) were obtained by multiplying 70 years for 365 days (Liu *et al.*, 2006).

3. Results and Discussion

3.1 Validation of methods

The validation method was based on five different parameters: calibration curve equation, correlation coefficients (R^2), relative standard deviation (RSD), detection limit (DL) and recovery rates for spiked wine samples (Rec), as shown in Tables 3.1, 3.4 and 3.6.

The linearity of the calibration curve was evaluated by the proximity of R^2 to 1, and is related with the accuracy of the concentrations determined. RSD values should be as small as possible once they determine the differences between the various measures of the same sample. Detection limit represents the lowest value quantified with efficiency for a measurement, and if the concentration found is below DL it should be discarded. Recovery rates allow determining the extent of trace elements quantified, comparing the known concentration added to a sample and the final concentration determined.

Experimental data obtained in the validation of methods for the quantification of Hg, Cd, Ni and Pb by HGAAS, ETAAS and FAAS in the presence of H_2O_2 are shown in Table 3.1. The results for As quantification are presented later because sample treatments were different. Validation process included the analysis of wines with and without acid digestion. In the last column of Table 3.1, WD represents samples not submitted to digestion and WAD deals with samples that have been digested.

Table 3.1:Experimental data obtained for method validation of Hg, Cd, Ni and Pb in the presence of H_2O_2 and with or without previous acid digestion.

Trace element	Methodology	Calibration curve equation	R^2	RSD %	DL	Rec %
Hg	HGAAS	y=0.002910x+0.008900	0.999	2.800-8.000	0.5193 μgL^{-1}	3170-13590 (WD)
						2310-6930 (WAD)
Cd	FAAS	y=0.210996x-0.008273	0.995	1.042-11.04	0.0458 mgL^{-1}	51.80-109.6 (WD)
						0.4427-2.158 (WAD)
	ETAAS	y=0.012625x+0.066130	0.991	0.405-2.025	2.6401 μgL^{-1}	206.7-519.2 (WD)
						180.2-477.5 (WAD)
Ni	FAAS	y=0.032024x+0.000636	0.999	0.724-18.64	0.0424 mgL^{-1}	100.9-155.3 (WD)
						10.87-112.1 (WAD)
Pb	FAAS	y=0.00697x+0.002116	0.998	0.898-13.44	0.0919 mgL^{-1}	56.30-117.2 (WD)
						46.79-59.30 (WAD)
	ETAAS	y=0.004828x+0.046968	0.991	0.927-10.16	2.7811 μgL^{-1}	32.11-46.05 (WD)
						32.06-49.44 (WAD)

The smallest R^2 was of 0.991 for ETAAS technique used for Cd and Pb quantification. Values above 0.990 indicate that the fit of the calibration curve is satisfactory, so besides being the lowest R^2 value is still an acceptable value (Edgerley, 1998). RSD percentage was calculated from three consecutive determinations of each sample. The highest values of RSD were obtained for FAAS, as it is the less sensitive technique used, due to the variations of measurements. DL was higher for FAAS and the explanation given above for RSD values is also valid for DL. The recovery rates (Rec) were calculated from the samples to which a known concentration of trace elements was added (Tables 3.2 and 3.3), dividing the concentration determined by the concentration of spike in the wine and multiplying the result for 100. Higher recovery rates for samples without acid digestion is a common element to all the techniques performed except for Pb quantification by ETAAS. In this case, the difference between recovery percentages from digested and non-digested wine samples is insignificant. The analysis of data shown in Table 3.1 shows that there was no need for this pre-digestion step of the Portuguese red wines studied in this work. This fact is clearly shown when comparing the concentrations present in Tables 3.2 and 3.3.

The concentrations of trace elements obtained in six different Portuguese red wines, in the presence of H_2O_2, with and without acid digestion, are shown in Table 3.2. HGAAS was used to perform Hg quantification; ETAAS was the technique selected for Cd quantification; FAAS technique was used in the quantification of Ni and Pb.

Table 3.2: Concentrations of Hg, Cd, Ni and Pb in the presence of H_2O_2, in six Portuguese red wines used in the method validation phase.

Wine	Concentration of Hg (μgL^{-1})		Concentration of Cd (μgL^{-1})		Concentration of Ni (mgL^{-1})		Concentration of Pb (mgL^{-1})	
	Without digestion	With acid digestion	Without digestion	With acid digestion	Without digestion	With acid digestion	Without digestion	With acid digestion
A1	2.129	<DL	<DL	<DL	<DL	0.182	<DL	<DL
A2	2.865	<DL	<DL	<DL	<DL	0.060	<DL	<DL
A1s	2.647	6.543	12.22	12.58	2.243	1.570	2.190	<DL
A2s	3.847	9.238	11.56	3.400	1.207	0.137	1.723	<DL
B1	2.972	<DL	<DL	<DL	<DL	<DL	<DL	<DL
B2	2.917	0.770	<DL	<DL	<DL	0.139	<DL	<DL
B1s	5.506	0.770	5.953	<DL	0.787	0.624	<DL	<DL
B2s	7.621	<DL	5.727	<DL	0.417	0.870	<DL	<DL
C1	2.381	<DL	<DL	<DL	<DL	<DL	<DL	<DL
C2	1.863	3.078	<DL	3.2345	<DL	<DL	<DL	<DL
C1s	2.670	1.155	7.633	<DL	0.523	0.478	<DL	<DL
C2s	2.516	1.925	4.210	10.45	0.760	0.687	<DL	<DL
D1	1.328	<DL	<DL	<DL	<DL	0.047	<DL	<DL

Table 3.2: Continued.

Wine	Concentration of Hg (μgL^{-1})		Concentration of Cd (μgL^{-1})		Concentration of Ni (mgL^{-1})		Concentration of Pb (mgL^{-1})	
	Without digestion	With acid digestion	Without digestion	With acid digestion	Without digestion	With acid digestion	Without digestion	With acid digestion
D2	1.805	<DL	<DL	<DL	<DL	0.043	<DL	<DL
D1s	3.090	3.848	4.540	<DL	0.747	0.567	<DL	<DL
D2s	3.927	9.797	8.963	<DL	0.140	0.880	<DL	<DL
E1	1.729	3.078	<DL	<DL	<DL	0.072	<DL	<DL
E2	1.799	4.233	<DL	<DL	<DL	<DL	<DL	<DL
E1s	2.246	1.925	13.97	<DL	0.653	0.537	<DL	<DL
E2s	2.558	0.770	3.557	<DL	0.697	0.520	<DL	<DL
F1	1.777	<DL	<DL	<DL	<DL	0.078	<DL	<DL
F2	1.316	<DL	<DL	<DL	<DL	<DL	<DL	<DL
F1s	10.57	<DL	13.18	10.39	0.693	0.333	<DL	<DL
F2s	11.24	<DL	10.38	10.18	0.540	1.057	<DL	<DL

s: spiked wine.

Table 3.3 shows the concentrations obtained for the quantification of Pb and Cd by ETAAS and FAAS, respectively. The six different Portuguese red wines were analyzed in the presence of H_2O_2, with and without acid digestion.

Table 3.3: Concentrations obtained for Cd and Pb in the presence of H_2O_2, with and without acid digestion.

Wine	Concentration of Pb by ETAAS (μgL^{-1})		Concentration of Cd by FAAS (mgL^{-1})	
	Without digestion	With acid digestion	Without digestion	With acid digestion
A1	<DL	<DL	<DL	0.054
A2	<DL	<DL	<DL	0.075
A1s	<DL	<DL	0.183	<DL
A2s	<DL	<DL	0.092	<DL
B1	<DL	<DL	<DL	0.112
B2	<DL	<DL	<DL	0.069
B1s	<DL	<DL	<DL	<DL
B2s	<DL	<DL	<DL	<DL
C1	<DL	<DL	<DL	0.063
C2	<DL	<DL	<DL	0.100
C1s	<DL	<DL	7.633	<DL
C2s	<DL	<DL	4.210	<DL
D1	<DL	<DL	<DL	0.079
D2	<DL	<DL	<DL	0.111

Table 3.3: Continued.

Wine	Concentration of Pb by ETAAS (μgL^{-1})		Concentration of Cd by FAAS (mgL^{-1})	
	Without digestion	With acid digestion	Without digestion	With acid digestion
D1s	<DL	<DL	<DL	<DL
D2s	<DL	<DL	<DL	<DL
E1	<DL	<DL	<DL	0.100
E2	<DL	<DL	<DL	0.059
E1s	<DL	<DL	<DL	<DL
E2s	<DL	<DL	<DL	<DL
F1	<DL	<DL	<DL	<DL
F2	<DL	<DL	<DL	<DL
F1s	<DL	<DL	<DL	<DL
F2s	<DL	<DL	<DL	<DL

s: spiked wine

The results obtained for the non-digested wine samples (Tables 3.2 and 3.3) were better for non-spiked samples and even much better for spiked ones, being the recovery rates substantially higher than those obtained in the digested wine samples. These results allow quantifying the trace elements with lower costs and with less time consuming techniques.

From the concentrations obtained for all the trace elements in study, Pb represents the chemical element with less concentration of all trace elements studied. From the concentrations obtained for FAAS (Table 3.2) and ETAAS (Table 3.3), it is clear that the first technique was the most adequate to quantify Pb in the studied wines. Differently from what was referred in the "Introduction" section, it seems that the new materials used in the equipments for wine production are not a motive of concern on wine contamination with Pb.

Cd was also quantified by two different techniques: ETAAS (Table 3.2) had shown better results for this trace element. With this technique, concentrations of 14 μgL^{-1} were found in the wine samples analyzed with spike and without acid digestion. Being Cd one of the trace elements that are subjected to bioaccumulation, it is mainly accumulated in soil and vineyard.

Hg concentration was never above 11.5 μgL^{-1} but on the other hand, Ni was quantified in high concentrations, being the highest value of 2.2 mgL^{-1}, in both cases for spiked samples and without acid digestion (Table 3.2).

After analyzing the pre-digesting process in the recovery and quantification efficiencies of the trace elements and after the definition of the most adequate quantification technique, the same

experimental procedures were performed without the presence of H_2O_2. Table 3.4 shows the experimental data obtained in the quantification of Hg, Cd, Ni and Pb by HGAAS, ETAAS and FAAS in the absence of H_2O_2 and without a previous acid digestion process.

Table 3.4:Experimental data obtained for method validation of Hg, Cd, Ni and Pb in the absence of H_2O_2 and without previous acid digestion.

Trace elements	Methodology	Calibration curve equation	R^2	RSD (%)	DL	Rec (%)
Hg	HGAAS	y=0.005100x+0.0102	0.995	1.600-15.30	0.03888 μgL^{-1}	1.864-22.07
Cd	ETAAS	y=0.057503x+0.0241	0.990	0.149-2.278	1.16547 μgL^{-1}	25.25-51.91
Ni	FAAS	y=0.084827x-0.0021	0.999	0.624-4.680	0.03261 mgL^{-1}	83.85-100.2
Pb	FAAS	y = 0.007000x+0.0021	0.998	0.408-10.08	0.08394 mgL^{-1}	34.13-39.10

The recovery rates calculated for samples without H_2O_2 (Table 3.4) were lower than that obtained for wine samples without digestion in the presence of H_2O_2 (Table 3.1). In fact, worse results than those given by pre-acidic digestion were even obtained (Table 3.1).

The concentrations obtained to calculate recovery rates are shown in Table 3.5. It can be concluded that Portuguese red wines analyzed needed the presence of H_2O_2 to oxidize the matrix and allow trace elements to be free for quantification. The decay of the values is obvious when comparing the concentration obtained for trace elements without hydrogen peroxide (Table 3.5) with those obtained in the presence of H_2O_2 (Tables 3.2 and 3.3).

Table 3.5: Concentrations obtained for Hg, Cd, Ni and Pb without H_2O_2.

Wine	Concentration of Hg (μgL^{-1})	Concentration of Cd (μgL^{-1})	Concentration of Ni (mgL^{-1})	Concentration of Pb (mgL^{-1})
A1	<DL	<DL	<DL	<DL
A2	<DL	<DL	<DL	<DL
A1s	<DL	<DL	<DL	<DL
A2s	<DL	<DL	<DL	<DL
B1	<DL	<DL	<DL	<DL
B2	1.157	<DL	<DL	<DL
B1s	<DL	<DL	<DL	<DL
B2s	0.621	<DL	<DL	<DL
C1	<DL	<DL	<DL	<DL
C2	0.372	<DL	<DL	<DL
C1s	<DL	<DL	<DL	<DL
C2s	<DL	<DL	<DL	<DL
D1	<DL	<DL	<DL	<DL
D2	0.171	<DL	<DL	<DL
D1s	<DL	<DL	<DL	<DL

Table 3.5: Continued.

Wine	Concentration of Hg (μgL^{-1})	Concentration of Cd (μgL^{-1})	Concentration of Ni (mgL^{-1})	Concentration of Pb (mgL^{-1})
D2s	<DL	<DL	<DL	<DL
E1	<DL	<DL	<DL	<DL
E2	0.736	<DL	<DL	<DL
E1s	<DL	<DL	<DL	<DL
E2s	<DL	<DL	<DL	<DL
F1	<DL	<DL	<DL	<DL
F2	0.337	<DL	<DL	<DL
F1s	<DL	<DL	<DL	<DL
F2s	<DL	<DL	<DL	<DL

s: spiked wine

Because As quantification required different approaches in order to perform a precise quantification, the results are shown separately from the previous trace elements. Wine samples with and without acid digestion, and with and without spike addition were analyzed. As well the addition of KI (pre-reducer agent) and an evaporation in water bath (Table 3.6) were also performed. All determinations were performed with the addition of H_2O_2.

Table 3.6: Experimental data obtained for As method validation by four different sample preparation methods.

Sample preparation	Calibration curve equation	R^2	RSD (%)	DL ($\mu g\ L^{-1}$)	Rec (%)
Without digestion	y=0.00387x-0.0114	0.992	1.200-12.30	0.0005	0.001-0.015
Acid digestion	y=0.00149x+0.0054	0.998	6.900-15.80	0.0014	0.000-4.000
Evaporation	y=0.0008x-0.0021	1.000	5.600-13.40	1.408	1365-3374
KI addition	y=0.00203x-0.0001	0.991	7.800-15.30	0.9541	ND

ND: not determined.

The smallest R^2 was of 0.991 when KI was added to wine samples not digested. On the other hand, R^2 value for evaporated samples (1.000) was the highest. The recovery rates observed for As were very low for non-evaporated samples. The maximum Rec value found was of 4% for samples without acid digestion. Those values were calculated from the concentrations obtained for all sample treatments techniques, except for KI addition, showed in Table 3.7. After evaporation, Rec values increased for percentages above 3300%.

Table 3.7: Concentrations obtained for As in six different Portuguese red wines by HGAAS.

Wine	Concentration of As without digestion (μgL^{-1})	Concentration of As with acid digestion (μgL^{-1})	Concentration of As evaporated (μgL^{-1})	Concentration of As with KI (μgL^{-1})
A1	<DL	0.0080	<DL	1.189
A2	<DL	0.0063	<DL	<DL
A1s	<DL	0.0087	<DL	ND
A2s	<DL	0.0077	<DL	ND
B1	<DL	<DL	<DL	4.289
B2	<DL	<DL	<DL	2.097
B1s	<DL	<DL	5.421	ND
B2s	<DL	<DL	<DL	ND
C1	0.0173	0.0087	<DL	6.435
C2	<DL	0.0123	<DL	4.185
C1s	<DL	0.0097	8.889	ND
C2s	<DL	0.016	2.901	ND
D1	<DL	<DL	<DL	4.425
D2	<DL	<DL	<DL	<DL
D1s	<DL	<DL	<DL	ND
D2s	<DL	<DL	6.4596	ND
E1	<DL	<DL	<DL	<DL
E2	0.0010	<DL	<DL	<DL
E1s	<DL	<DL	5.890	ND
E2s	<DL	<DL	13.46	ND

Table 3.7: Continued.

Wine	Concentration of As without digestion (μgL^{-1})	Concentration of As with acid digestion (μgL^{-1})	Concentration of As evaporated (μgL^{-1})	Concentration of As with KI (μgL^{-1})
F1	<DL	<DL	<DL	4.190
F2	<DL	<DL	<DL	2.546
F1s	<DL	<DL	15.41	ND
F2s	<DL	<DL	10.92	ND

ND: not determined.
s: spiked wine.

Low As concentrations were obtained in wine samples that were non-digested as can be seen in Table 3.7. Only for two wines it was possible to quantify a concentration of As that was below 0.01 μgL^{-1}. It can be concluded that with this technique As is not free for quantification, which is confirmed by the low recovery rates (Table 3.6). As was quantified in a higher number of digested wine samples than in non-digested ones. The highest As content was below 0.02 μgL^{-1}. In this case, Rec was of 4%, which strenghts the fact that this is not the adequate technique for As quantification.

KI was added to wine samples not spiked and not digested for As quantification (Table 3.7). This technique proved to be more efficient than the quantification in digested or non-digested samples. However, KI contaminated the AAS equipment, being not possible to keep performing this technique. The quantification of As after a previous evaporation of wine samples in a water bath at 40ºC allowed ethanol to evaporate. This reduces considerably the interference of ethanol in As quantification. But, due to the temperature used in the digestion process, this would be also accomplished by "Digiprep" system. Nevertheless, since the Rec values were low after the digestion process, it must be concluded that ethanol was not the only interference, or at least it was not the most significant. In the evaporation technique, the oxidation state of As is not affected as it may occur in the acid digestion. Table 3.6 shows high Rec values associated to the evaporation pre-treatment, which conducted to the choice of this technique as the correct one for As quantification in Portuguese red wines.

From the As concentrations obtained by HGAAS, despite the samples preparation, the highest value was obtained with evaporation, but was below 15.5 μgL^{-1} (Table 3.7).

From the analysis performed was determined that Ni and Pb quantification was performed with FAAS, Cd determined with ETAAS, and Hg and As were quantified bu using HGAAS. All the

trace elements in study, except As, were analyzed without sample preparation. For As quantification the wine samples were evaporated previously.

3.2 Trace elements in Portuguese red wines

The concentrations of trace elements in non-spiked wine samples representative of eleven IG Portuguese regions are shown in Table 3.8. All wine samples were analyzed without spike (Table 3.8) and with spike (Table 3.9) to determine if the addition of a known concentration of each metal was essential for the detection of trace elements.

Table 3.8: Concentrations of trace elements in Portuguese red wines for non-spiked wine samples.

Wine origin	Ni concentration (mgL^{-1})	Pb concentration (mgL^{-1})	Cd concentration (μgL^{-1})	Hg concentration (μgL^{-1})	As concentration (μgL^{-1})
Alentejo	<DL	<DL	<DL	2.129	<DL
Alentejo	<DL	<DL	<DL	2.865	<DL
Alentejo	<DL	<DL	<DL	2.972	<DL
Alentejo	<DL	<DL	<DL	2.917	<DL
Alentejo	<DL	<DL	<DL	2.381	<DL
Alentejo	<DL	<DL	<DL	1.863	<DL
Alentejo	<DL	<DL	<DL	1.709	10.377
Alentejo	<DL	<DL	2.166	0.256	10.000
Alentejo	<DL	<DL	<DL	0.684	10.000
Alentejo	<DL	<DL	<DL	0.342	9.906
Alentejo	<DL	<DL	<DL	<DL	4.088
Alentejo	<DL	<DL	<DL	<DL	4.403
Algarve	1.058	<DL	<DL	<DL	1.100
Algarve	1.049	<DL	<DL	<DL	1.106
Beiras	1.031	<DL	<DL	<DL	1.015
Beiras	1.089	<DL	<DL	<DL	0.937
Beiras	<DL	<DL	0.504	<DL	3.385
Beiras	<DL	<DL	0.584	<DL	2.308
Beiras	<DL	<DL	3.537	<DL	11.692
Beiras	<DL	<DL	4.334	<DL	9.538
Dão	<DL	<DL	<DL	0.533	7.231
Dão	<DL	<DL	<DL	0.833	7.538
Douro	<DL	<DL	<DL	<DL	<DL
Douro	<DL	<DL	<DL	<DL	<DL
Douro	<DL	<DL	2.722	<DL	6.981

Table 3.8: Continued.

Wine origin	Ni concentration (mgL^{-1})	Pb concentration (mgL^{-1})	Cd concentration (μgL^{-1})	Hg concentration (μgL^{-1})	As concentration (μgL^{-1})
Douro	<DL	<DL	2.541	<DL	10.566
Douro	<DL	<DL	43.485	0.598	7.925
Douro	<DL	<DL	48.378	0.513	2.264
Douro	<DL	<DL	<DL	0.725	4.277
Douro	<DL	<DL	<DL	0.797	2.923
Estremadura	<DL	<DL	<DL	<DL	<DL
Estremadura	<DL	<DL	<DL	<DL	<DL
Estremadura	1.050	<DL	<DL	2.137	1.282
Estremadura	1.101	<DL	<DL	1.892	1.135
Madeira	<DL	<DL	<DL	<DL	0.943
Madeira	<DL	<DL	<DL	<DL	0.629
Minho	<DL	<DL	<DL	<DL	4.923
Minho	<DL	<DL	<DL	<DL	2.615
Minho	<DL	<DL	<DL	<DL	9.748
Minho	<DL	<DL	<DL	<DL	11.321
Ribatejo	1.094	<DL	<DL	6.787	2.952
Ribatejo	1.179	<DL	<DL	2.317	2.339
Ribatejo	<DL	<DL	5.950	0.342	10.377
Ribatejo	<DL	<DL	4.214	0.855	10.566
Terras do Sado	<DL	<DL	<DL	<DL	<DL
Terras do Sado	<DL	<DL	<DL	<DL	<DL
Terras do Sado	1.094	<DL	<DL	<DL	<DL
Terras do Sado	1.063	<DL	<DL	<DL	<DL
Trás-os-Montes	<DL	<DL	<DL	<DL	5.975
Trás-os-Montes	<DL	<DL	<DL	<DL	5.031

The concentrations obtained for spiked wine samples, for all IG Portuguese regions that produce red wine, are represented in Table 3.10.

Table 3.9: Concentrations obtained for spiked wine samples representative of IG Portuguese regions.

Wine origin	Ni concentration (mgL^{-1})	Pb concentration (mgL^{-1})	Cd concentration (μgL^{-1})	Hg concentration (μgL^{-1})	As concentration (μgL^{-1})
Alentejo	2.243	1.507	12.220	2.647	7.832
Alentejo	1.207	1.037	11.560	3.847	<DL
Alentejo	0.787	<DL	5.953	5.507	5.421
Alentejo	0.417	<DL	5.727	7.621	<DL
Alentejo	0.523	<DL	7.633	2.670	8.890
Alentejo	0.760	<DL	4.210	2.516	2.901
Alentejo	0.113	<DL	1.846	3.335	12.617
Alentejo	0.313	<DL	17.686	3.335	6.201
Alentejo	<DL	<DL	39.061	3.165	12.428
Alentejo	0.317	<DL	43.898	3.079	11.862
Alentejo	0.280	<DL	3.520	<DL	13.183
Alentejo	0.323	<DL	6.597	<DL	14.440
Algarve	2.940	<DL	6.253	2.446	1.493
Algarve	2.050	<DL	6.513	1.613	1.277
Beiras	2.460	<DL	5.080	1.445	0.892
Beiras	2.527	<DL	8.423	3.743	2.271
Beiras	0.613	<DL	3.761	<DL	5.513
Beiras	0.403	<DL	4.747	<DL	4.590
Beiras	0.328	<DL	8.835	0.460	10.898
Beiras	0.547	<DL	3.698	0.569	9.667
Dão	0.453	<DL	17.429	0.786	9.513
Dão	0.453	<DL	19.393	0.388	9.975
Douro	0.693	<DL	13.177	10.568	15.411
Douro	0.540	<DL	10.377	11.242	10.915
Douro	0.397	<DL	26.254	2.652	9.409
Douro	0.073	<DL	26.237	3.079	11.484
Douro	<DL	<DL	61.045	3.592	11.296
Douro	<DL	<DL	40.143	3.677	9.220

Table 3.9: Continued.

Wine origin	Ni concentration (mgL^{-1})	Pb concentration (mgL^{-1})	Cd concentration (μgL^{-1})	Hg concentration (μgL^{-1})	As concentration (μgL^{-1})
Douro	0.817	<DL	1.840	0.569	5.775
Douro	0.923	<DL	1.329	0.605	6.421
Estremadura	0.653	<DL	13.973	2.246	5.890
Estremadura	0.697	<DL	3.557	2.558	13.455
Estremadura	2.173	<DL	6.810	2.317	4.097
Estremadura	2.040	<DL	5.967	6.249	1.415
Madeira	0.167	<DL	0.530	0.413	10.981
Madeira	0.257	<DL	1.657	0.341	11.296
Minho	0.403	<DL	2.418	0.315	6.334
Minho	0.567	<DL	5.672	0.460	3.421
Minho	0.277	<DL	7.980	0.874	16.956
Minho	0.263	<DL	10.017	0.590	18.528
Ribatejo	1.413	<DL	6.200	3.687	3.774
Ribatejo	1.400	<DL	6.410	2.242	2.695
Ribatejo	0.160	<DL	36.941	2.994	12.428
Ribatejo	0.173	<DL	49.499	3.677	11.862
Terras do Sado	0.747	<DL	4.540	3.090	<DL
Terras do Sado	0.140	<DL	8.963	3.927	6.460
Terras do Sado	2.723	<DL	6.473	1.692	2.237
Terras do Sado	2.797	<DL	6.757	1.562	1.370
Trás-os -Montes	0.803	<DL	12.437	<DL	11.296
Trás-os -Montes	0.600	<DL	16.407	<DL	12.239

For all the trace elements the number of wines in which it was possible to detect metals above the Detection Limits (DL) increased significantly after the addition of a known concentration of metals.

Pb content in spiked and non-spiked wine samples was below the DL, except for one spiked wine sample and for its duplicate. Pb is the only trace element in wine that has a maximum level established in the European Regulation (CE) nº 1881/2006, which is used as a reference for Portuguese wines. This limit value is of 200 μgL^{-1} (Table 1.4). OIV has a more restrictive level which is defined at 150 μgL^{-1}. Both values were exceeded by the two wine samples in which Pb concentration was detected.

The trace element for which it was possible a broader quantification in non-spiked wine samples was As. Nevertheless, concentrations of As for spiked wine samples as for non-spiked samples were below the limit value defined by OIV of 200 μgL^{-1}.

In non-spiked wine samples, Cd concentrations were much higher than the maximum level defined by OIV (10 μgL^{-1}), being reached the concentration of 48 μgL^{-1}. Cd concentrations for wine samples with spike reached 49 μgL^{-1}. Since the concentrations found are four times higher than the established by OIV, it can be concluded that the Cd concentration in some of the Portuguese red wines analyzed are a motive of concern.

Since OIV has not defined maximum limits for Hg and Ni in wines, the Ni concentrations determined were compared to Tolerable Upper Intake Levels (defined by The National Academy Press, 2006) and Hg concentrations were compared to Provisional Tolerable Weekly Intakes (FAO/WHO, 2010) values defined for adults (1 mg kg^{-1} day^{-1} for Ni and 1.6 μg kg^{-1} $week^{-1}$ for Hg).

In order to compare the Ni and Hg concentrations found in Portuguese red wines with these limit values, it was necessary to express the concentrations as a function of the body weight (kg) and exposure time (days or weeks). In Table 3.11 and 3.12 are shown the Daily Intake Levels for spiked and non-spiked samples, for both men and women. The body weights considered for each gender wer of 75.9 kg for men and of 63 kg for women. The daily amount of red wine ingested for both gender were considered to be of 1.96 drinks per day, being one drink equivalent to a volume of 250 mL glass wine (Eurobarometer of the European Comission, 2003; Naughton & Petróczi, 2008).

Table 3.10: Ni and Hg Daily Intake Levels and Weekly Intake Levels respectively for men (M) and women (W) for non-spiked wine samples.

Wine origin	Ni_M (mg kg^{-1} d^{-1})	Ni_W (mg kg^{-1} d^{-1})	Hg_M (µg kg^{-1} $week^{-1}$)	Hg_W (µg kg^{-1} $week^{-1}$)
Alentejo	ND	ND	0.1295	0.1548
Alentejo	ND	ND	0.1343	0.1605
Alentejo	ND	ND	0.1318	0.1576
Alentejo	ND	ND	0.1076	0.1286
Alentejo	ND	ND	0.0842	0.1006
Alentejo	ND	ND	0.0772	0.0923
Alentejo	ND	ND	0.0116	0.0138
Alentejo	ND	ND	0.0309	0.0369
Alentejo	ND	ND	0.0155	0.0185
Alentejo	ND	ND	ND	ND
Alentejo	ND	ND	ND	ND
Alentejo	ND	ND	ND	ND
Algarve	0.0068	0.0082	ND	ND
Algarve	0.0068	0.0081	ND	ND
Beiras	0.0067	0.0080	ND	ND
Beiras	0.0070	0.0084	ND	ND
Beiras	ND	ND	ND	ND
Beiras	ND	ND	ND	ND
Beiras	ND	ND	ND	ND
Beiras	ND	ND	0.0241	0.0288
Dão	ND	ND	0.0376	0.0450
Dão	ND	ND	ND	ND
Douro	ND	ND	ND	ND
Douro	ND	ND	ND	ND
Douro	ND	ND	ND	ND
Douro	ND	ND	0.0270	0.0323
Douro	ND	ND	0.0232	0.0277
Douro	ND	ND	0.0328	0.0392
Douro	ND	ND	0.0360	0.0431
Douro	ND	ND	ND	ND
Estremadura	ND	ND	ND	ND
Estremadura	ND	ND	0.0966	0.1154
Estremadura	0.0068	0.0081	0.0855	0.1022
Estremadura	0.0071	0.0085	ND	ND
Madeira	ND	ND	ND	ND
Madeira	ND	ND	ND	ND
Minho	ND	ND	ND	ND
Minho	ND	ND	ND	ND
Minho	ND	ND	ND	ND

Table 3.10: Continued.

Wine origin	Ni_M (mg kg^{-1} d^{-1})	Ni_W (mg kg^{-1} d^{-1})	Hg_M (µg kg^{-1} $week^{-1}$)	Hg_W (µg kg^{-1} $week^{-1}$)
Minho	ND	ND	0.3067	0.3666
Ribatejo	0.0071	0.0084	0.1047	0.1252
Ribatejo	0.0076	0.0091	0.0155	0.0185
Ribatejo	ND	ND	0.0386	0.0462
Ribatejo	ND	ND	ND	ND
Terras do Sado	ND	ND	ND	ND
Terras do Sado	ND	ND	ND	ND
Terras do Sado	0.0071	0.0084	ND	ND
Terras do Sado	0.0069	0.0082	ND	ND
Trás-os-Montes	ND	ND	ND	ND
Trás-os-Montes	ND	ND	ND	ND

ND: not determined.

Ni, for non-spiked wine samples, was found in Daily Intake Level under 0.008 mg kg^{-1} day^{-1} for men, and slightly higher Daily Intake Levels for women that reached 0.0091 mg kg^{-1} day^{-1}. The values are much lower than 1 mg kg^{-1} day^{-1} advised by The National Academy Press. Hg for both genders never exceeded the Weekly Intake Level of 1.6 µg kg^{-1} $week^{-1}$, and was also higher for women. Being the Weekly Intake Level intake defined at 1.6 µg kg^{-1} $week^{-1}$ there is no motif for concern.

Table 3.11:Ni and Hg Daily Intake Levels and Weekly Intake Levels respectively for men (M) and women (W) for spiked wine samples.

Wine origin	Ni_M (mg kg^{-1} day^{-1})	Ni_W (mg kg^{-1} d^{-1})	Hg_M (µg kg^{-1} $week^{-1}$)	Hg_W (µg kg^{-1} $week^{-1}$)
Alentejo	0.0145	0.0173	0.1196	0.1430
Alentejo	0.0078	0.0093	0.1739	0.2078
Alentejo	0.0051	0.0061	0.2489	0.2974
Alentejo	0.0027	0.0032	0.3444	0.4117
Alentejo	0.0034	0.0040	0.1207	0.1442
Alentejo	0.0049	0.0059	0.1137	0.1359
Alentejo	0.0007	0.0009	0.1507	0.1802
Alentejo	0.0020	0.0024	0.1507	0.1802
Alentejo	ND	ND	0.1430	0.1709
Alentejo	0.0020	0.0024	0.1391	0.1663
Alentejo	0.0018	0.0022	ND	ND
Alentejo	0.0021	0.0025	ND	ND
Algarve	0.0190	0.0227	0.1106	0.1321
Algarve	0.0132	0.0158	0.0729	0.0871
Beiras	0.0159	0.0190	0.0653	0.0781
Beiras	0.0163	0.0195	0.1692	0.2022

Table 3.11: Continued.

Wine origin	Ni_M (mg kg^{-1} d^{-1})	Ni_W (mg kg^{-1} d^{-1})	Hg_M (µg kg^{-1} $week^{-1}$)	Hg_W (µg kg^{-1} $week^{-1}$)
Beiras	0.0040	0.0047	ND	ND
Beiras	0.0026	0.0031	ND	ND
Beiras	0.0021	0.0025	0.0208	0.0249
Beiras	0.0035	0.0042	0.0257	0.0307
Dão	0.0029	0.0035	0.0355	0.0425
Dão	0.0029	0.0035	0.0175	0.0209
Douro	0.0045	0.0054	0.4776	0.5709
Douro	0.0035	0.0042	0.5081	0.6073
Douro	0.0026	0.0031	0.1198	0.1432
Douro	0.0005	0.0006	0.1391	0.1663
Douro	ND	ND	0.1623	0.1940
Douro	ND	ND	0.1662	0.1986
Douro	0.0053	0.0063	0.0257	0.0307
Douro	0.0060	0.0071	0.0273	0.0327
Estremadura	0.0042	0.0050	0.1015	0.1213
Estremadura	0.0045	0.0054	0.1156	0.1382
Estremadura	0.0140	0.0168	0.1047	0.1252
Estremadura	0.0132	0.0157	0.2824	0.3375
Madeira	0.0011	0.0013	0.0187	0.0223
Madeira	0.0017	0.0020	0.0154	0.0184
Minho	0.0026	0.0031	0.0142	0.0170
Minho	0.0037	0.0044	0.0208	0.0249
Minho	0.0018	0.0021	0.0395	0.0472
Minho	0.0017	0.0020	0.0267	0.0319
Ribatejo	0.0091	0.0109	0.1666	0.1992
Ribatejo	0.0090	0.0108	0.1013	0.1211
Ribatejo	0.0010	0.0012	0.1353	0.1617
Ribatejo	0.0011	0.0013	0.1662	0.1986
Terras do Sado	0.0048	0.0058	0.1397	0.1669
Terras do Sado	0.0009	0.0011	0.1775	0.2121
Terras do Sado	0.0176	0.0210	0.0765	0.0914
Terras do Sado	0.0181	0.0216	0.0706	0.0844
Trás-os-Montes	0.0052	0.0062	ND	ND
Trás-os-Montes	0.0039	0.0046	ND	ND

ND: not determined.

Similarly to non-spiked samples, for spiked wine samples Ni and Hg have shown Daily and Weekly Intake Levels below 1 mg kg^{-1} d^{-1} and 1.6 µg kg^{-1} $week^{-1}$, respectively.

3.3 Statistical analysis

The statistical analysis was only performed on the data obtained for spiked wine samples, due to the low number of wines in which the metals were quantified above the detection limits.

The statistical analysis held in this work was restricted to As, Hg, Cd and Ni. Pb was excluded because it was only quantified in two wine samples and all the concentrations that were determined below the DL interfered negatively in the statistical analysis of the system, making the statistical software unable to deliver data. To ensure data accuracy it was also defined that only the regions with more than one wine analyzed would be considered in the statistical analysis. Therefore, the following regions were considered in the statistical analysis: Alentejo, Beiras, Douro, Estremadura, Minho, Ribatejo and Terras do Sado.

Figure 3.1 represents maximum and minimum concentrations and median values Hg, Cd and As. Ni is not represented because the concentrations were determined in mgL^{-1} instead of μgL^{-1}, which would lead to the impossibility to analyse with efficiency the values of the other three trace elements if Ni was present in the Figure 3.1.

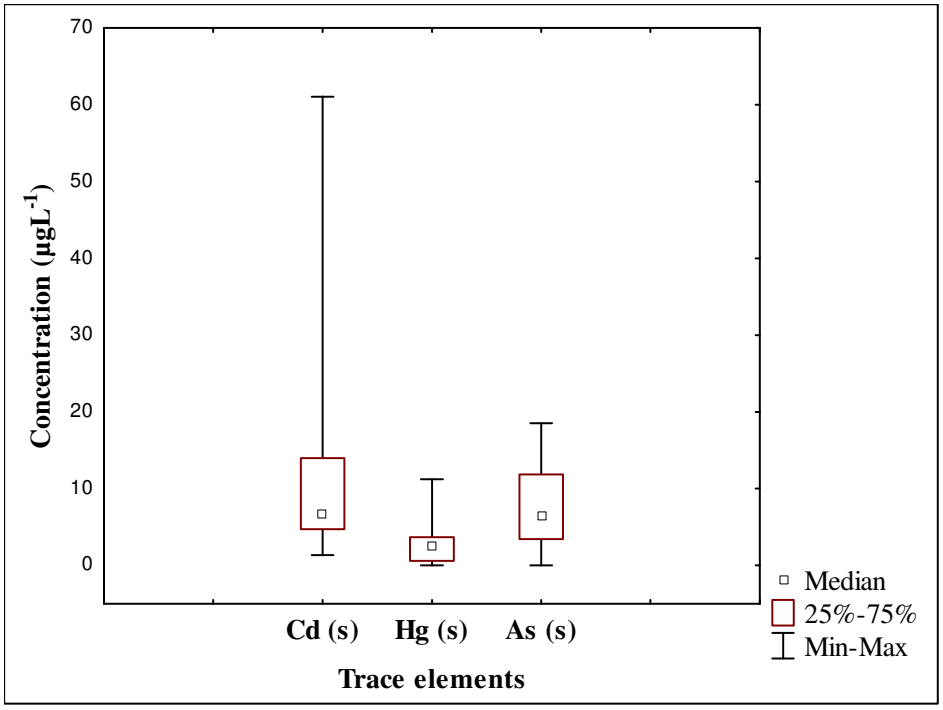

Figure 3.1: Box & Whisker plot for Ni, Cd, Hg and As.

Figure 3.1 shows that the median values for Hg was low, which is coherent with the low concentrations determined. The median values for As and Cd were higher than for Hg, but they had associated a broader range of the interval comprising 25% to 75% of the As and Cd concentrations determined in the wines. Cd presented the major amplitude of concentrations determined in the wines when maximum and minimum values were taken into consideration.

PCA analysis determined the Portuguese regions that have homogeneous distribution for a certain trace element. Homogeneous distribution means that a group of regions have a mean concentration for a trace element that is within the concentrations range defined by the "STATISTICA 8" software. Depending on the concentrations variance the software groups the regions in two or three different groups (Table 3.12 to Table 3.15). Table 3.13 shows the Portuguese wine regions with higher contents of Ni.

Table 3.12: Least Significant Difference test for Ni as variable.

Region	Ni mean	1	2	3
Minho	0.377	****	****	
Douro	0.430	****		
Alentejo	0.607	****	****	
Ribatejo	0.787	****	****	
Beiras	1.146	****	****	****
Estremadura	1.391		****	****
Terras do Sado	1.841			****

****: represents an homogenous group of regions for Ni distribution.

Mean values of Cd distribution for the seven Portuguese wine producing regions are represented in Table 3.13.

Table 3.13: Least Significant Difference test for Cd as variable.

Region	Cd mean	1	2	3
Beiras	5.757	****		
Minho	6.521	****	****	****
Terras do Sado	6.698	****	****	
Estremadura	7.577	****	****	****
Alentejo	13.33	****	****	****
Douro	22.55			****
Ribatejo	24.76		****	****

****: represents an homogenous group of regions for Cd distribution.

Hg mean values were determined by PCA analysis for all the Portuguese regions under statistical analysis. Table 3.14 shows the distribution of Hg content in Portuguese red wines along the country.

Table 3.14: Least Significant Difference test for Hg as variable.

Region	Hg mean	1	2
Minho	0.559	****	
Beiras	1.036	****	
Terras do Sado	2.367	****	****
Alentejo	3.144	****	****
Ribatejo	3.150	****	****
Estremadura	3.343	****	****
Douro	4.498		****

****: represents an homogenous group of regions for Hg distribution.

Statistical analysis allowed to determine the homogeneous distribution for As in Portuguese regions (Table 3.15).

Table 3.15: Least Significant Difference test for As as variable.

Region	As mean	1	2
Terras do Sado	2.287	****	
Beiras	5.639	****	
Estremadura	6.215	****	****
Ribatejo	7.689	****	****
Alentejo	7.981	****	****
Douro	9.991	****	****
Minho	11.31		****

****: represents an homogenous group of regions for As distribution.

By using the one-way ANOVA analysis, it was possible to define the distribution of the trace elements along the seven Portuguese wine producing regions, for a 0.95 confidence interval. This analysis made possible to conclude the following: Ni was present in higher concentrations in wines of Estremadura and Terras do Sado (Table 3.12); Cd was largely present in Douro and Ribatejo wines (Table 3.13); Hg concentrations were higher in Douro wines (Table 3.14); the highest concentrations of As were determined in Minho wines (Table 3.15).

PCA determined four factors to explain the system variance, being the system variance composed by the four trace elements and the seven Portuguese wine producing regions in study. Eigenvalue are representative of the importance of each factor in the total system variance (Table 3.16). The software produced four factors because there are four variables, As, Hg, Cd and Ni.

Table 3.16: Eigenvalues of correlation matrix, and related statistics.

Factor	Eigenvalue	% Total variance	Cumulative eigenvalue	Cumulative %
1	1.935	48.37	1.935	48.37
2	1.050	26.26	2.985	74.63
3	0.639	15.96	3.624	90.59
4	0.376	9.407	4.000	100.0

PCA produced a number of factors equal to the number of variables in the system. In this case, they were four: As, Hg, Cd and Ni. As shown in Table 3.16, the factors 1 and 2 have associated a total variance of 75% of the system, which means that the majority of the variance of the system can be explained only by these two factors. The eigenvalues of factors 1 and 2 are above one, showing the highest contribution of these factors for the system variance. Factors 3 and 4 had a variance sum of only 25%, and both eigenvalues were below 1, which lead to the exclusion of these factors from the system variance.

The Factor Coordinates represented in Table 3.17 alowed to determine the impact of each factor in each trace element (Ni, Cd, Hg and As). There are two types of factors, positive and negative, which indicate if the relation between the trace element concentrations and factors are directly or inversely proportional, respectively.

Table 3.17: Factor coordinates of the variables, based on correlations.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Ni	-0.846	0.187	0.246	-0.435
Cd	0.700	0.288	0.653	-0.003
Hg	0.166	0.936	-0.310	-0.022
As	0.837	-0.237	-0.237	-0.432

The variable contributions (Table 3.17) made possible to know the trace elements that contribute largely for each factor. Ni and As were the most significant trace elements for factor 1, despite of having different signals. Factor 2 was influenced almost by Hg. Therefore, the global system variation can be explained mainly by the concentration variations of As, Ni and Hg, which were associated to factors 1 and 2.

In Table 3.18 are presented the correlations between the four trace elements, this correlation allows to determine if the appearance of a certain trace element is related to the appearance or absence of another trace element.

Table 3.18: Correlations between four trace elements in study.

Variable	Ni	Cd	Hg	As
Ni	1.000	-0.377	-0.032	-0.623
Cd	-0.377	1.000	0.183	0.365
Hg	-0.032	0.183	1.000	-0.001
As	-0.623	0.365	-0.001	1.000

The values obtained for correlations between the trace elements in study showed a slight influence between the presence of As and Ni. Since the value of correlation is negative it is possible to conclude that whenever one of these two metals are present there is a probability that the other is not present.

3.4 Target Hazard Quotients

THQ values were calculated through equation 1.1. They were represented in the following Figures by the median value, the range comprising 25% and 75% of the calculated THQ values and the range comprising the minimum and maximum THQ value for each gender. THQ values were calculated based on the concentrations of trace elements determined in spiked wine samples (identified with the "s" letter) and non-spike wine samples.

Figure 3.2 shows the THQ values of As, Hg, Cd, Ni and Pb calculated for Alentejo region and for both gender.

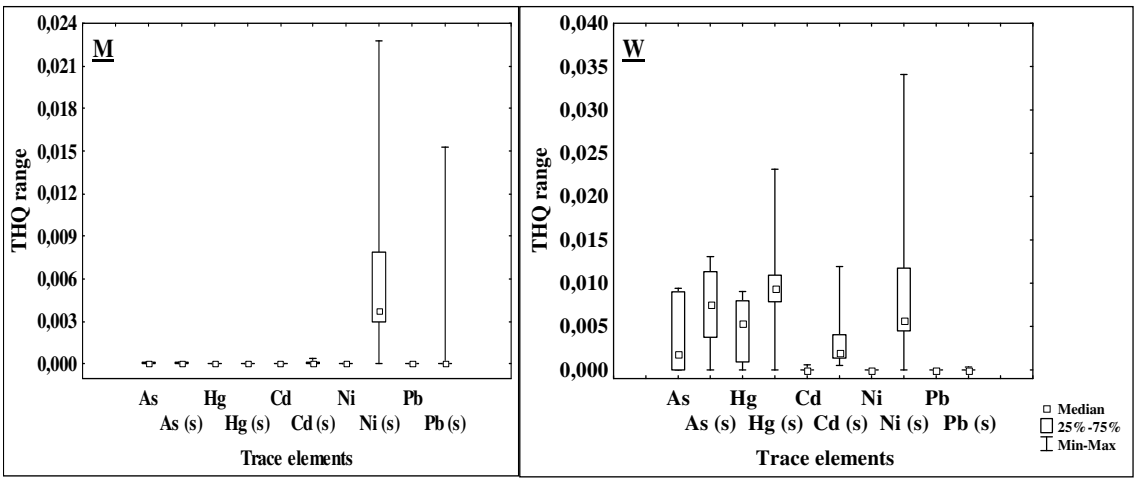

Figure 3.2: THQ values for Alentejo wines for men (M) and women (W) (s: spiked wines).

THQ values for Algarve wines are represented in Figure 3.3.

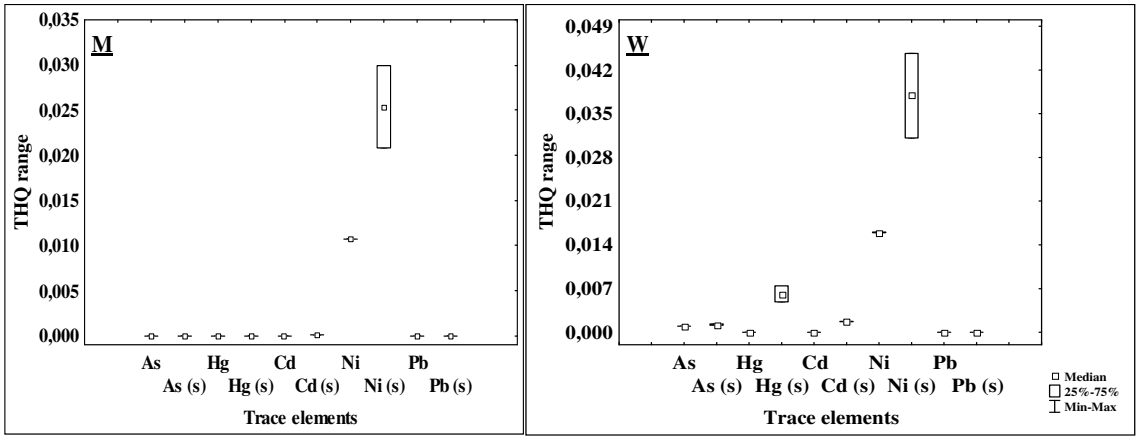

Figure 3.3: THQ values for Algarve wines for men (M) and women (W) (s: spiked wines).

THQ values for Beiras wines are shown in Figure 3.4.

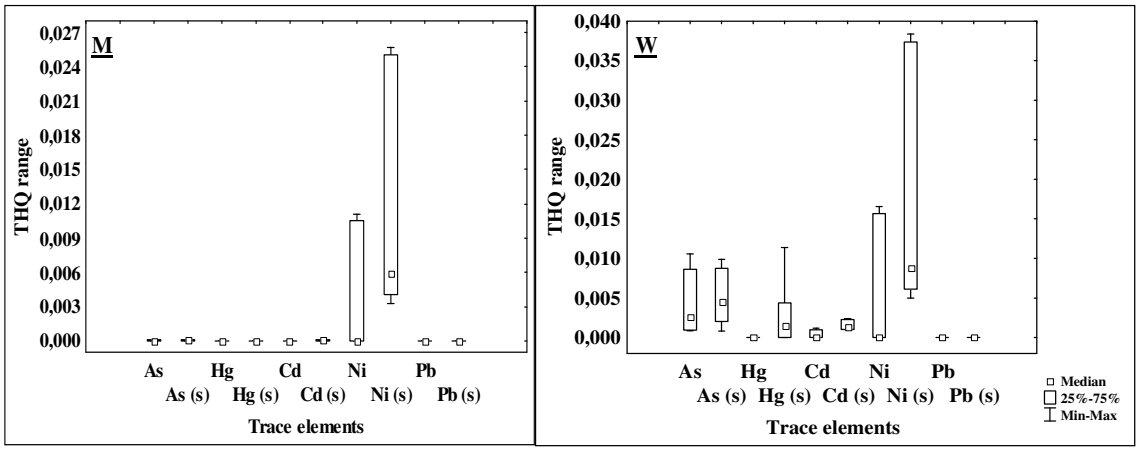

Figure 3.4: THQ values for Beiras wines for men (M) and women (W) (s: spiked wines).

Dão THQ values for Portuguese red wines are represented in Figure 3.5.

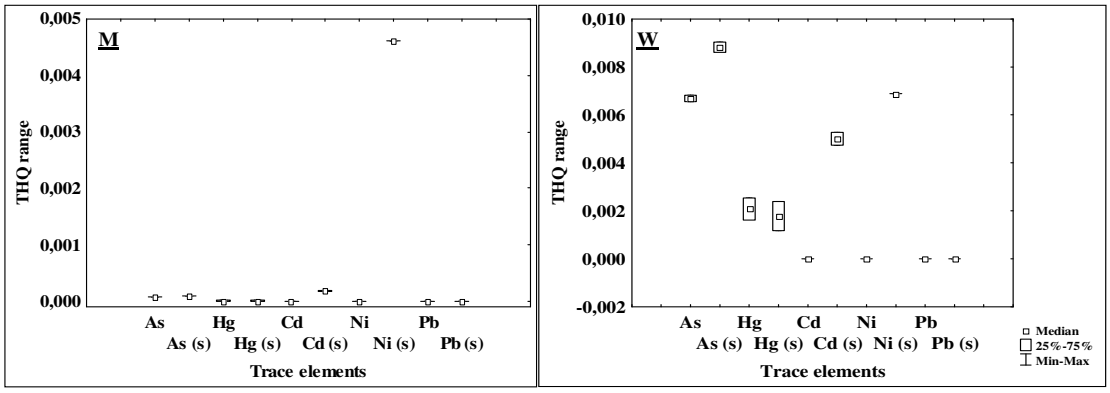

Figure 3.5: THQ values for Dão wines for men (M) and women (W) (s: spiked wines).

Douro THQ values for Portuguese red wines are represented in Figure 3.6.

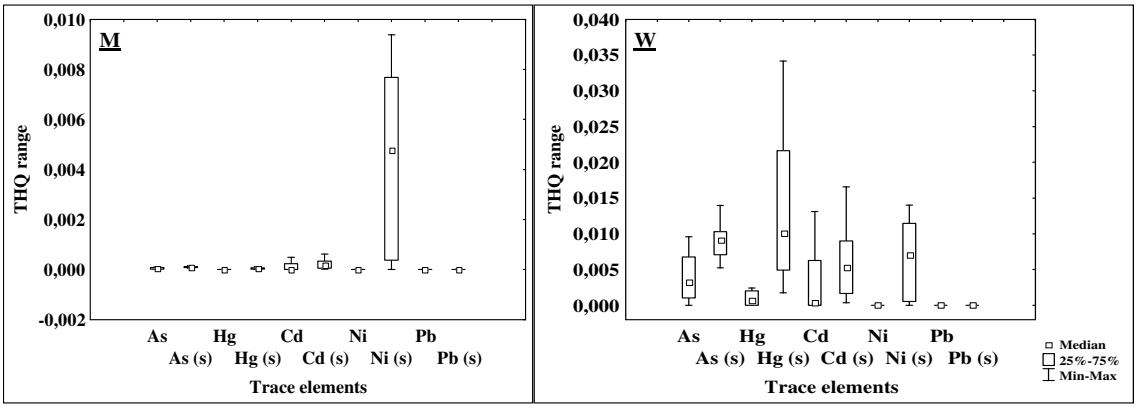

Figure 3.6: THQ values for Douro wines for men (M) and women (W) (s: spiked wines) .

THQ values for Estremadura wines are shown in Figure 3.7.

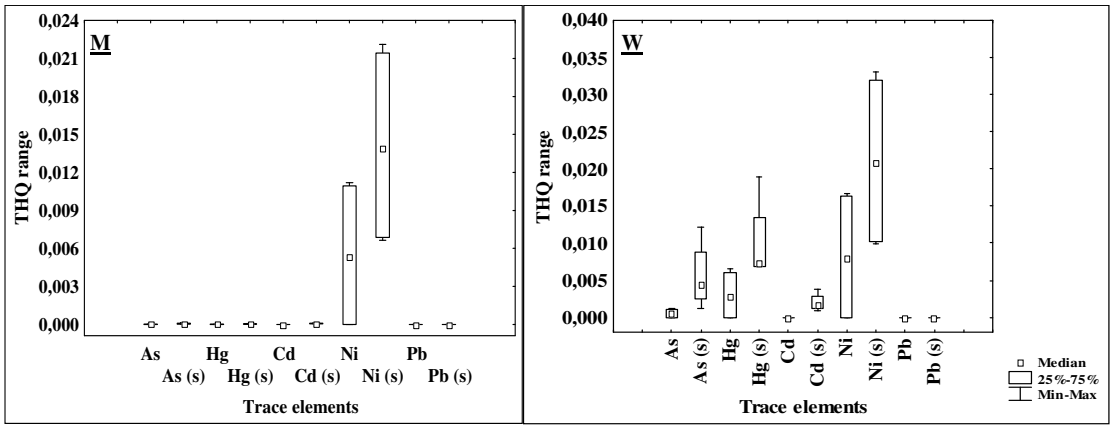

Figure 3.7: THQ values for Estremadura wines for men (M) and women (W) (s: spiked wines).

THQ values of Madeira region is presented in Figure 3.8.

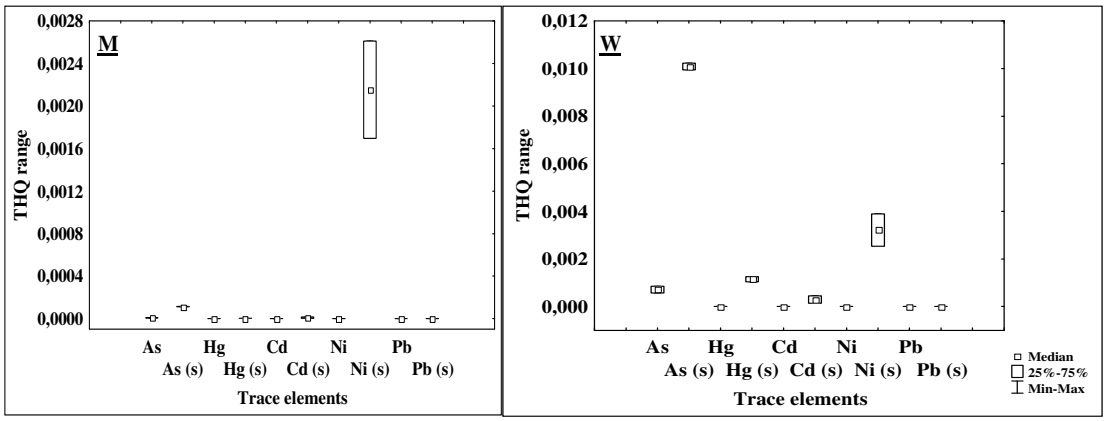

Figure 3.8: THQ values for Madeira wines for men (M) and women (W) (s: spiked wines).

In Figure 3.9 are represented THQ values for Minho wines.

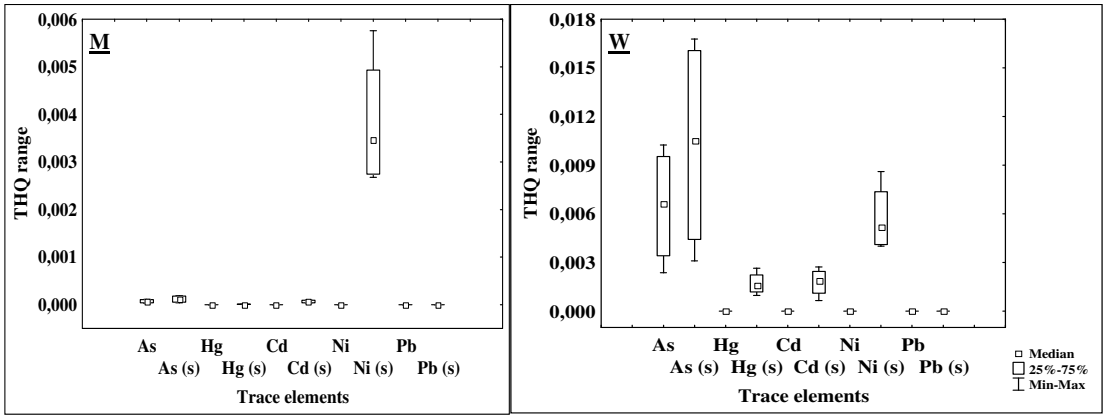

Figure 3.9: THQ values for Minho wines for men (M) and women (W) (s: spiked wines).

In Figure 3.10 are represented THQ values for Ribatejo wines.

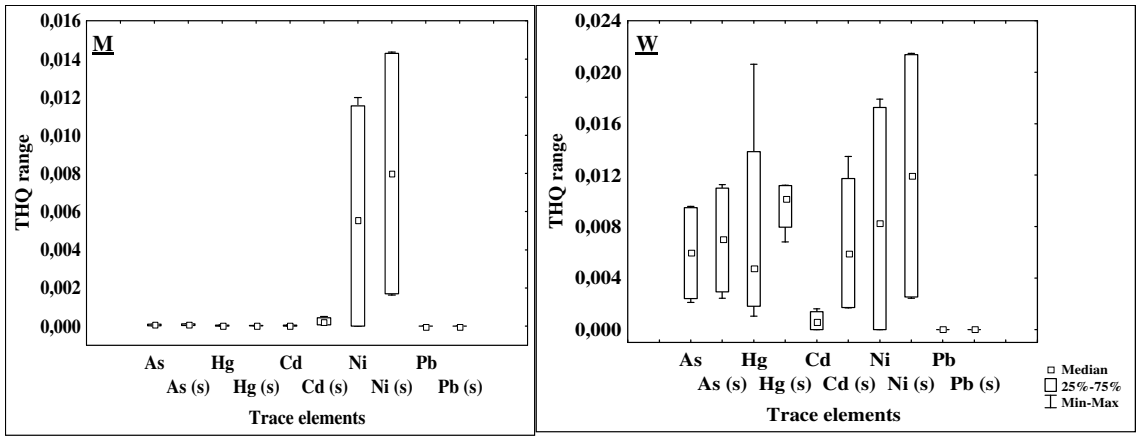

Figure 3.10: THQ values for Ribatejo wines for men (M) and women (W) (s: spiked wines).

THQ values for Trás-os-Montes wines are shown in Figure 3.11

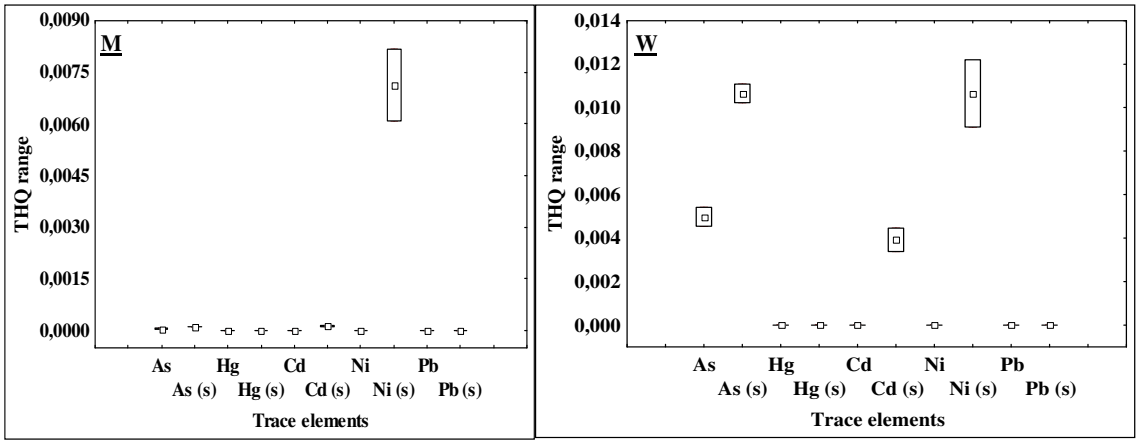

Figure 3.11: THQ values for Trás-os-Montes wines for men (M) and women (W) (s: spiked wines).

In Figure 3.12 are represented THQ values for Terras do Sado wines.

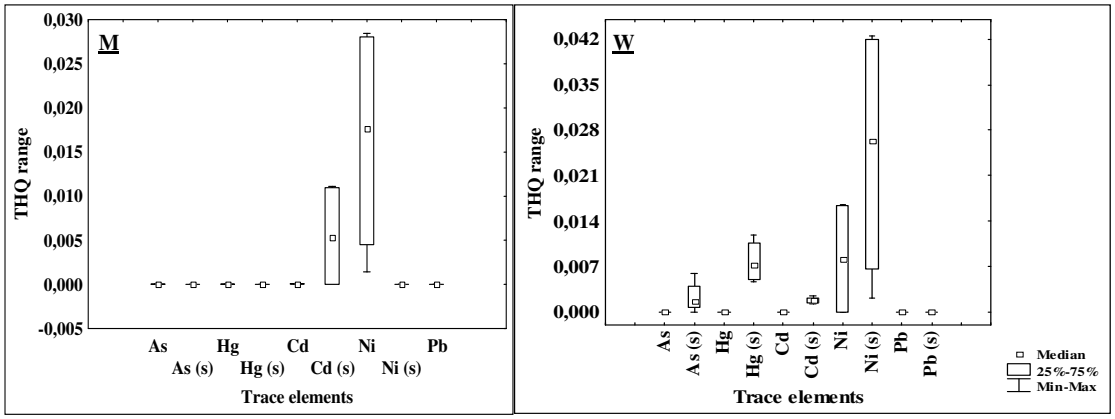

Figure 3.12: THQ values for Terras do Sado wines for men (M) and women (W) (s: spiked wines).

Maximum THQ values for Alentejo were 0.034 for women and 0.0228 for men. For both genders Ni was responsible for highest values (Figure 3.2).

Maximum THQ values in Algarve wines were due to Ni, and were of 0.044 and 0.029 for women and men, respectively. The THQ value for women was the highest value of all quotients determined in the wines of this region (Figure 3.3).

For Beiras wines, Ni was responsible for the broader THQ range, which for women reached the value of 0.0374 and for men of 0.0257 (Figure 3.4).

The wines of Dão region revealed different THQ distributions between genders (Figure 3.5). For women, THQ values reached 0.009 due to As. For men, THQ values were distributed attaining a value of 0.0046 for Ni.

The highest quotients relative to Douro wines were for women 0.034, due to Hg, and due to Ni for men the values reached 0.0094 (Figure 3.6).

In Estremadura wines, Ni was once again responsible for the highest THQ values, reaching 0.033 for women and 0.022 for men (Figure 3.7).

Madeira quotients were up to 0.0102 for women because of As concentrations. For men, THQ values reached values of 0.0026 due to Ni (Figure 3.8).

THQ values determined for Minho wines were high for women due to As, which reached a value of 0.0168, and in men gender the value of 0.0058 was reached due to Ni concentrations (Figure 3.9).

THQ highest values determined for Ribatejo wines (Figure 3.10) were due to Ni. The quotients reached a value of 0.022 for women and 0.014 for men.

The THQ values determined in Trás-os-Montes wines for Ni were of 0.012 for women, while for men the values were around 0.008 (Figure 3.11).

The THQ values for Terras do Sado (Figure 3.12) were higher for women, being Ni the responsible for these THQ values. For women THQ were up to 0.043 and for men reached 0.008.

All THQ values determined were, for all regions and both genders, lower than 1, which indicates a that there is not a motif for health concern.

4. Conclusions

The aim of this thesis is to characterize the composition of some Portuguese red wines in terms of five trace elements: As, Hg, Cd, Ni and Pb. First of all, it was necessary to validate the methodologies to be used in order to guarantee the quantification accuracy. In this part of the study, different sample treatments were tested. It was determined that besides As the other four trace elements wouldn't need a pre-treatment to enable an accurate quantification. On the other hand, for an accurate quantification of As in Portuguese red wines it was necessary a previous evaporation in a water bath. With this technique a recovery rate of 3300% was achieved. The techniques chosen for the quantification were HGAAS for As and Hg, ETAAS for Cd and FAAS for Ni and Pb.

Afterwards, 25 Portuguese red wines and their duplicates were analyzed with the methodologies previously chosen. Pb concentrations were below the limit value of 150 μgL^{-1} defined by OIV. Therefore, the concentrations of this trace element were not considered to be of concern for consumers' health. On the other hand, Cd had shown concentrations above the limit value of 10 μgL^{-1} defined by OIV .

It was also possible to quantify As in concentrations above 15 μgL^{-1} in evaporated wine samples. Nonetheless, As content in the studied Portuguese red wines was below the limit value of 200 μgL^{-1} defined by OIV.

In what concerns Hg and Ni, their Daily Intake Levels (DIL) was compared with Tolerable Upper Intake Levels and Provisional Tolerable Weekly Intake defined by FAO/WHO (2010) and The National Academy Press (2006), respectively, because OIV has not defined up to now limit values for both these trace elements. The Portuguese red wines analyzed in this work showed that Hg concentrations for spiked and non-spiked samples were always below the DIL of 1.6 $\mu g\ Kg^{-1}\ week^{-1}$, and Ni was below the DIL of 1 $mg\ Kg^{-1}\ d^{-1}$ for both genders.

The ANOVA one-way analysis made possible to conclude that As was the trace element showing the highest concentrations in Minho wines, while Hg was mainly present in Douro wines, Ni in Estremadura and Minho wines and Cd in Douro and Ribatejo wines.

PCA analysis made possible to conclude that two main factors have explained 75% of the concentration variance of four trace elements analyzed (As, Hg, Ni and Cd) in red wines of seven wine producing regions (Alentejo, Beiras, Douro, Estremadura, Minho, Ribatejo and

Terras do Sado). Ni, As and Hg were the trace elements mainly associated to these two factors and explained the majority of the system variance.

The highest value of THQ was 0.044, which was found for Algarve wines when Ni was the trace element analyzed. For men, the highest THQ values were found in Algarve, Beiras and Terras do Sado red wines, as for women the highest THQ values were determined for Algarve, Terras do Sado and Beiras red wines. The values never exceeded 1, so for this determination, there was not a motif for health concern of wine consumers.

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